Immunoprecipitation and the High Sensitivity Protein 250 Assay

Combining Specific and Sensitive Detection of Proteins with the Agilent 2100 Bioanalyzer

Application Note

Protein Electrophoresis

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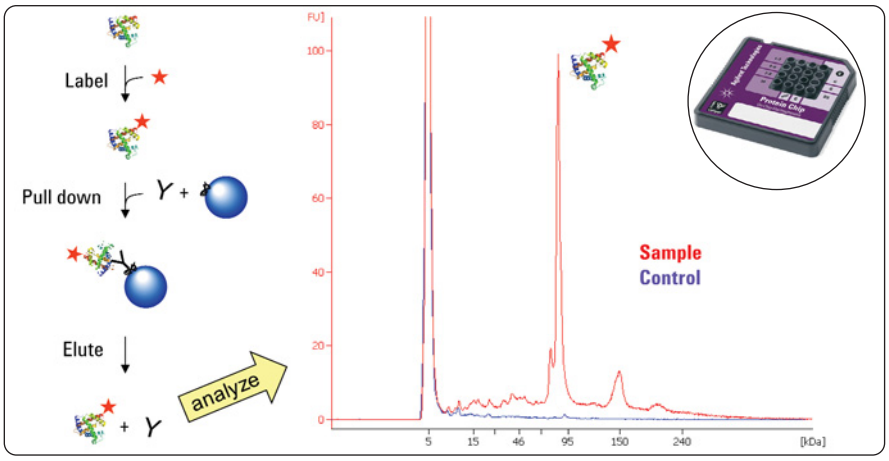

Abstract

A new method for the targeted analysis of proteins is presented that combines the specificity of immunoprecipitation with the sensitivity of protein detection on microchips using the High Sensitivity Protein 250 assay for the Agilent 2100 Bioanalyzer. As as an alternative to Western blotting, this method is valuable for researchers engaged, for example, in protein expression and purification in pharma, biotech or academic labs. Advantages of this new method in comparison to Western blotting are:

- More reliable results: higher specificity and sensitivity
- Better accuracy and precision: less manual steps and direct availability of quantitative data
- Increased productivity: 3 hours versus 1 day analysis time
- Lower spending for antibodies: 10x less primary and no secondary antibody are needed
- Lower reagent consumption: environmentally friendly process

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Introduction

Today, immunoaffinity is a crucial tool for the targeted analysis of proteins in complex samples. Techniques like Enzyme Linked Immunosorbent Assay (ELISA) and Western Blotting are widely used for a broad range of applications such as biomarker candidate verification in body fluids or clone selection for recombinant protein expression. The Agilent 2100 Bioanalyzer is a versatile analytical tool that allows characterization of diverse biological samples such as DNA, RNA, proteins and cells.[1] However, most current applications measure all analytes in a given size range, for example the Protein 80 assay that determines the size and quantity of all sample proteins from 5 to 80 kDa. This Application Note describes an application for the targeted analysis of proteins with the Agilent 2100 Bioanalyzer based on the High Sensitivity Protein 250 assay in combination with immunoprecipitation (IP/HSP250 method, Figure 1). The High Sensitivity Protein 250 assay achieves protein sizing and quantification with sensitivity superior to silver-stained SDS-PAGE and a linear dynamic range spanning up to four orders of magnitude.[2] Figure 1 shows the workflow of the IP/HSP250 method. Highlighted in yellow are steps of the High Sensitivity Protein 250 assay for sample preparation and final analysis on chip.[3] Reagents, microchips and protocols necessary for these steps are supplied with the High Sensitivity Protein 250 kit. The immunoprecipitation was done after sample preparation employing target specific antibodies and Protein A coated magnetic beads. Elution of immunocomplexes from the beads was done by heat denaturation in an SDS and DTT containing buffer supplied with the High Sensitivity Protein 250 kit. The eluted proteins are directly loaded onto microchips and analyzed automatically with the Agilent 2100 Bioanalyzer. Total time demand for the IP/HSP250 method is about 3 h. Conventional immunoprecipitation techniques that use SDS-PAGE with silver or Coomassie staining for protein detection, may have a problematic background, because molecular tools like antibodies and capture reagents are stained as well. This is not an issue with the IP/HSP250 method since the sample proteins are labeled with a fluorescent dye in the initial sample preparation step. Only these labeled species are detected after the immunoprecipitation by laser induced fluorescence detection on-chip.

The performance of the IP/HSP250 method is shown with *E. coli* cell lysate samples spiked with a GST-tagged protein and tag-specific antibodies. Western blots were prepared with the same samples and antibodies to benchmark the new method against an established technique for targeted protein detection.

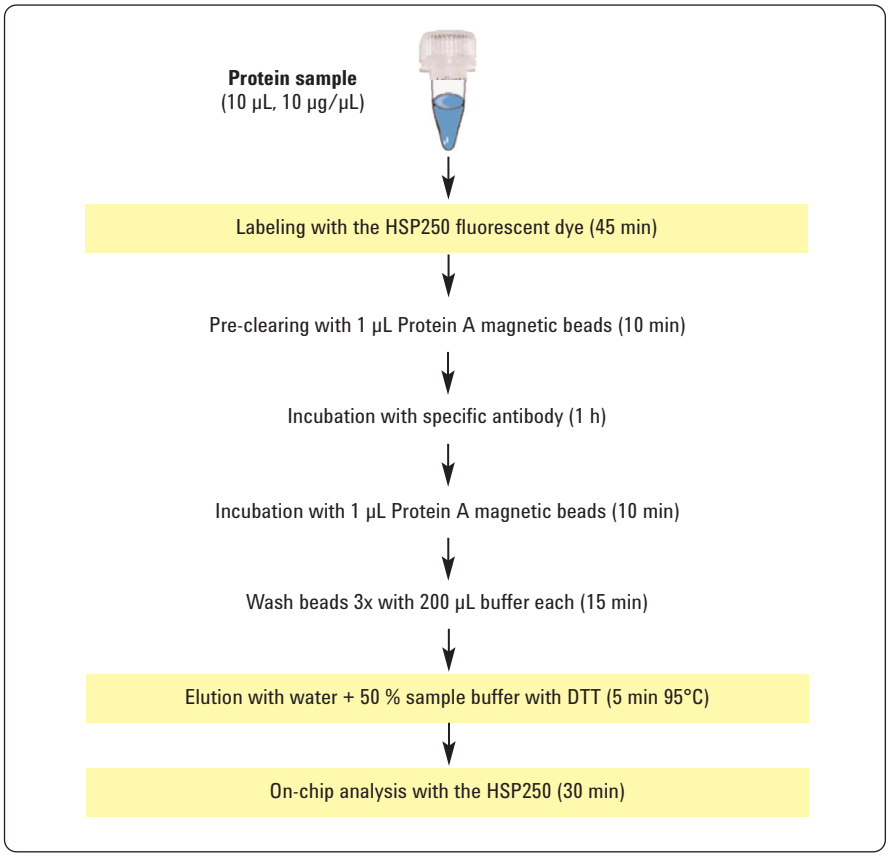

Figure 1
Workflow for the specific detection of tagged proteins with the IP/HSP250 method.

Experimental

Material

Dynabeads Protein A, DynaMag-2, XCell II Mini-Cell & Blot Module, precast 4-12 % BisTris minigels, prestained protein standard, PVDF membrane, buffers for SDS-PAGE and Western transfer, WesternBreeze Chromogenic Western Blot Detection Kit (Invitrogen, Carlsbad, CA, USA), Protein LoBind tubes (Eppendorf GmbH, Hamburg, Germany), Zeba Desalt Spin Columns and Coomassie Plus Assay Reagent (Pierce, Rockford, IL, USA), lyophilized *E. coli* strain B cells (ATCC 11303), rabbit polyclonal anti-GST antibody (Sigma, Taufkirchen, Germany), GST-tagged Phosphatase and tensin homolog (PTEN), Agilent 2100 bioanalyzer and High Sensitivity Protein 250 kit (Agilent Technologies GmbH, Waldbronn, Germany).

Sample preparation

GST-tagged PTEN was supplied as 1 mg/mL solution in 50 mM Tris/HCl pH 7.5, 50 mM NaCl, 1 mM EDTA and 5 mM DTT. Lyophilized *E. coli* cells were resuspended in 50 mM sodium phosphate buffer pH 8, 300 mM NaCl and disrupted with a BeadBeater (Biospec Products, Bartlesville, OK, USA). Cell debris was removed by centrifugation at 12,000 g for 10 min. A buffer exchange to 50 mM sodium phosphate buffer pH 8.3, 300 mM NaCl was done with 2 mL Zeba Desalt Spin Columns. Total protein concentration of the cell lysate was determined with the Coomassie Plus Assay Reagent.

E. coli extract at a concentration of 10 µg/µL was spiked with 1 % of GST-tagged PTEN (corresponding to 100 ng/µL target protein). Serial 10-fold dilutions of this 1 % mixture with *E. coli* lysate were prepared to obtain mixtures with 0.1 % (10 ng/µL), 0.01 % (1 ng/µL), 0.001 % (100 pg/µL) and 0.0001 % (10 pg/µL) tagged protein, respectively. Control samples of target protein (without *E. coli* lysate) were diluted with labeling buffer (30 mM Tris/HCl, pH 8.5) to 100 ng/µL.

Protein Labeling

Protein labeling was done as described in the Agilent High Sensitivity Protein 250 Kit Guide.[3]

Immunoprecipitation

Immunoprecipitation was done in Protein LoBind tubes with 10 µL of labeled sample. Tween 20 was added to the sample to a final concentration of 0.1 %.

Initially, samples were pre-cleared with Protein A beads to reduce background. One µL of magnetic Protein A beads was washed twice with 100 µL of wash buffer (50 mM sodium phosphate, pH 8, 300 mM NaCl, 0.1 % Tween 20). Once washed, the beads were added to the sample and incubated for 10 min on ice. Tubes were vortexed every 2–3 min to keep the beads in suspension. After 10 min, the cleared supernatant was collected and the beads were discharged.

Second, samples were incubated with the tag-specific antibody. Anti-GST antibody was diluted 100 to 1 in wash buffer. One µL of the antibody dilution was added to the sample and the mixture was incubated for 1 h on ice.

Next, immunocomplexes were captured with Protein A beads: One µL of Protein A beads was washed twice with wash buffer and added to the sample. After 10 min incubation on ice with vortexing every 2–3 min, the supernatant was discharged. The beads were washed 3 times with 200 µL wash buffer. Extra care was taken during the last wash step to remove the remaining liquid completely and avoid carry-over of salt.

Finally, captured proteins were eluted from the beads: The beads were resuspended with 10 µL of water and 5 µL of High Sensitivity Protein 250 sample buffer with DTT to elute all bound protein. After heating to 95 °C for 5 min, samples were centrifuged for 30 s and applied directly (without dilution) to the microchip.

On-chip analysis

On-chip sample analysis was done as described in the Agilent High Sensitivity Protein 250 Kit Guide.[3] The High Sensitivity Protein 250 ladder was labeled and analyzed according to the standard procedure.

Western blot

According to supplier instructions, SDS-PAGE was performed with 4 % to 12 % BisTris minigels. Western transfer was completed onto PVDF membranes. Finally, chromogenic immunodetection was completed with BCIT/NBT substrate and anti-rabbit secondary antibodies coupled to alkaline phosphatase. Primary anti-GST antibody was diluted, 2000:1.

Results and discussion

The sensitivity and specificity of the IP/HSP250 method was investigated with an *E. coli* lysate spiked with a different amount of GST-tagged phosphatase and tensin homolog (PTEN). Samples were labeled with a fluorescent dye, and aliquots were analyzed with the High Sensitivity Protein 250 assay. No difference was observed between the samples (Figure 2A). Then, samples were immunoprecipitated with a polyclonal rabbit anti-GST antibody and magnetic Protein A beads. Immunocomplexes eluted from the beads were directly loaded onto microchips and analyzed with the Agilent 2100 Bioanalyzer. After subtraction of the negative control run the LOD (S/N > 3) was determined to 0.001 % or 100 pg/µL PTEN in a background of 10 µg/µL *E. coli* lysate (Figure 2B). Recovery of PTEN target protein out of the *E. coli* lysate was estimated to about 15 % from the quantitative data provided by the Agilent 2100 Bioanalyzer. The low background observed in the IP/HSP250 experiments is in part due to the pre-clearing step (Figure 1). Therefore, sample pre-clearing can be helpful to further improve the detection limit, especially for low concentrations of the target protein (data not shown). Electropherograms of the immunoprecipitated samples appear very similar to those of the target protein control, implying that no

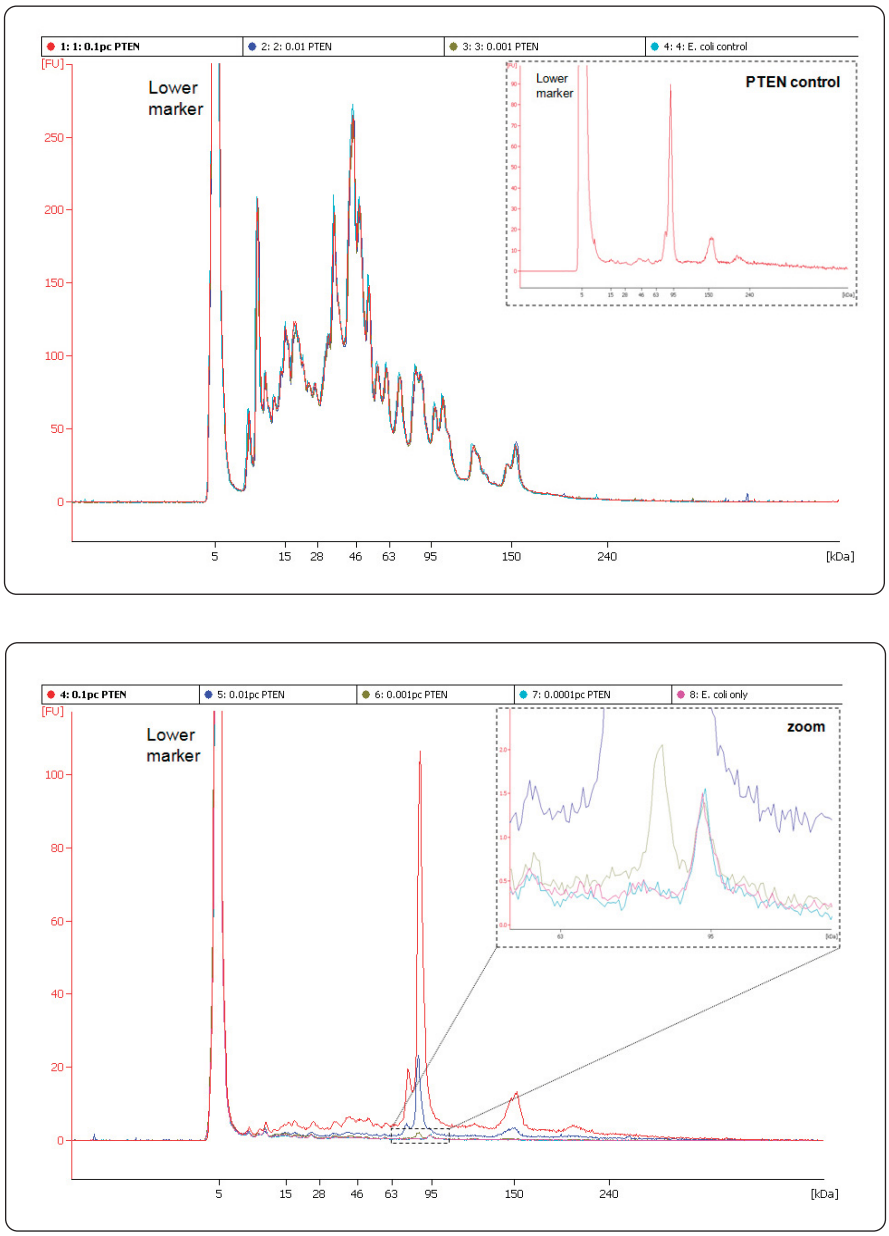

Figure 2
IP/HSP250 of *E. coli* lysate samples spiked with GST-tagged PTEN. (A) Analysis of samples after the labeling step. Overlaid are electropherograms of samples with 0.1 % (red), 0.01 % (blue), 0.001 % (green), and a negative control without PTEN (light blue). A direct analysis of PTEN with the High Sensitivity Protein 250 assay is shown in the inset ('PTEN control'). (B) Analysis of samples after immunoprecipitation. Overlaid are electropherograms of samples with 0.1 % (red), 0.01 % (blue), 0.001 % (green), 0.0001 % (light blue), and a negative control without PTEN (magenta). The inset shows a zoom on the PTEN main peak of the 0.001 % sample ('Zoom').

degradation or aggregation occurred during immunoprecipitation (Figure 2).

Western Blots were prepared for comparison. The same samples and tag-specific antibody were used as for the IP/HSP250 method (Figure 3). Blots for PTEN showed a high non-specific background present in all lanes, which is presumably due to the secondary anti-rabbit antibody used. A specific band was observed only at a concentration of 1 % or 100 ng/µL PTEN in a background of 10 µg/µL *E. coli* proteins (Figure 3, left panel). Thus, the IP/HSP250 method showed both higher sensitivity and specificity as compared to the Western blot, resulting in a 1,000 fold lower limit of detection of the target protein PTEN. Obviously, the risk of poor results due to low antibody quality is lower for the IP/HSP250 method with only a single antibody being used. Furthermore, the IP/HSP250 method allows antibody cost savings since consumption of primary antibody is at least 10 times lower than for Western blotting, and no secondary antibody is used. Additional advantages of the IP/HSP250 method over Western blotting are a significant faster time-to-result (3 h vs. one day), reduced manual

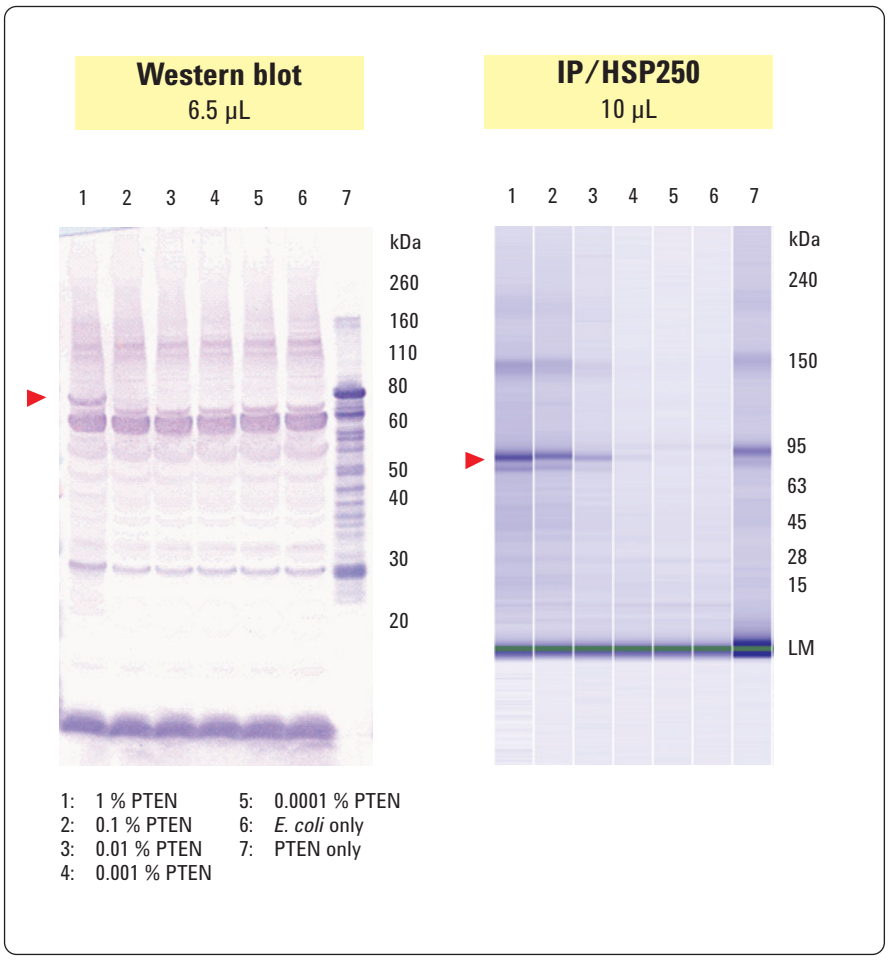

Figure 3
Comparison of the IP/HSP250 method to Western blotting with chromogenic immunocomplex detection for *E. coli* lysate samples spiked with GST-tagged PTEN. Sample volumes differed: 10 µL for IP/HSP250 vs. 6.5 µL for Western blotting. Shown are the Western blot (left) and the gel-like view of the High Sensitivity Protein 250 assay (right). The PTEN band is indicated with a red arrowhead. Molecular weights of standard proteins are shown (LM = lower marker).

intervention and the direct availability of quantitative data from the analysis with the Agilent 2100 Bioanalyzer.

Conclusion

The new IP/HSP250 method for the targeted detection of proteins delivered superior results in terms of specificity, sensitivity, usability, and time-to-result as compared to conventional Western Blotting with chromogenic immunocomplex detection. The method combines the specificity of immunoprecipitation with the high sensitivity of microfluidic protein detection on the Agilent 2100 Bioanalyzer.

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