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Qproteome FFPE Tissue Kit is not suitable for protein analysis using Agilent 2100 Bioanalyzer

Zastosowanie zestawu Qproteome FFPE Tissue Kit uniemożliwia analizę białek przy użyciu Bioanalizatora 2100

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Abstract

Introduction: The chip-based protein separation using Agilent 2100 Bioanalyzer is a promising tool for proteomic analysis. However, it has not been defined if the above-mentioned device is suitable for analysis of formalin-fixed paraffin-embedded (FFPE) proteins extracts.
The aim of the study: Therefore we performed the analysis aimed at testing if extracts from FFPE tissues are suitable for proteins detection using Agilent 2100 Bioanalyzer.
Material and methods: FFPE tissues and cell cultures were used for experiments. Proteins were extracted from them using Qproteome FFPE Tissue Kit and RIPA buffer, respectively. For protein analysis Bioanalyzer Instrument, Western Blot and immunohistochemistry (IHC) were applied.
Results: In FFPE extracts, using Bioanalyzer we failed to detect β-actin. However, Western Blot analysis proved β-actin presence in them. One of possible explanations of this phenomenon might be lack of antibody-antigen interaction during immunoprecipitation preceding Bioanalyzer analysis. We suspected that, Extraction Buffer (from Qproteome FFPE Tissue Kit) or β-mercaptoethanol (both present in FFPE protein extracts) might be responsible for the above-mentioned blockade. Therefore, we applied IHC with detection of CD34 because, CD34 staining is robust against small methodological variations and this marker is always present in tumor tissue sections. Anti-CD34 antibody was diluted in Tris saline buffer with Extraction Buffer, β-mercaptoethanol or without. We did not observe CD34 immunopositivity only in the presence of Extraction Buffer.
Conclusion: Qproteome FFPE Tissue Kit is not suitable for protein analysis using Agilent 2100 Bioanalyzer, because the Extraction Buffer from the kit prevents an interaction between antibody and antigen during immunoprecipitation procedure.

Streszczenie

Wprowadzenie: Bioanalizator Agilent 2100 jest narzędziem, które można wykorzystać w badaniach proteomicznych, jak dotąd nie sprawdzono jednak jego przydatności do analizy materiału utrwalonego w formalinie i zatopionego w parafinie (FFPE).
Cel badań: Dlatego celem przeprowadzonego badania było wykrycie β-aktyny w ekstraktach białkowych wyizolowanych z materiału FFPE przy użyciu Bioanalizatora Agilent 2100.
Materiał i metody: Do badania wykorzystano materiał z bloczków parafinowych oraz kultury tkankowe, z których otrzymano ekstrakty białkowe dzięki zastosowaniu, odpowiednio, zestawu Qproteome FFPE Tissue Kit oraz buforu RIPA. Analizę białek prowadzono wykorzystując bioanalizator, technikę Western Blot oraz immunohistochemię (IHC).
Wyniki: Podczas analizy z wykorzystaniem bioanalizatora w ekstraktach białkowych z FFPE nie wykryto β-aktyny, jednak potwierdzono jej obecność w tych samych ekstraktach za pomocą techniki Western Blot. Jedną z możliwych przyczyn tego zjawiska mogło być blokowanie tworzenia wiązania antygen-przeciwciało na etapie immunoprecypitacji (część procedury przygotowania próbki do analizy z użyciem bioanalizatora) przez bufor ekstrakcyjny (będący częścią zestawu Qproteome FFPE Tissue Kit) albo β-merkaptoetanol – oba obecne w białkowych ekstraktach FFPE. W celu sprawdzenia tej hipotezy przeprowadzono immunohistochemiczne barwienie CD34 (marker komórek endotelialnych), zawieszając przeciwciało pierwszorzędowe w roztworze z dodatkiem buforu ekstrakcyjnego lub β-merkaptoetanolu. Wybrano białko CD34, ponieważ, jak wynika z naszych wcześniejszych doświadczeń, jest zawsze wykrywane w tkankach nowotworowych, a procedura jego barwienia jest odporna na niewielkie zmiany metodologiczne. Nie obserwowano ekspresji markera (naczyń krwionośnych) jedynie na preparatach barwionych w obecności buforu ekstrakcyjnego.
Wnioski: Bufor ekstrakcyjny z zestawu Qproteome FFPE Tissue Kit blokuje wiązanie antygen-przeciwciało, dlatego jego użycie uniemożliwia analizę białek za pomocą Bioanalizatora Agilent 2100.

Key words: antigen-antibody binding, Agilent 2100 Bioanalyzer, immunohistochemistry, immunoprecipitation, protein extraction
Słowa kluczowe: wiązanie antygen-przeciwciało, Bioanalizator Agilent 2100, immunohistochemia, immunoprecypitacja, ekstrakcja białek

Introduction

The most popular tool for analysis of protein expression on formalin-fixed paraffin-embedded (FFPE) tissue sections is immunohistochemistry (IHC). However the resolution of IHC is too low to detect truncated and full length forms of particular protein, if they are situated in cell membrane of the same cell. The aforementioned situation might concern truncated form of HER2 protein, which can be generated at least by two independent mechanisms, i.e. proteolytic shedding or/and alternative initiation of translation [1]. In this case electrophoretic methods seem to be suitable, because they give the possibility to detect both two forms of HER2 and relative differences of their expression. Truncated proteins, including p95HER2 (transmembrane and intracellular domain of HER2 of 95-100 kDa) might have significant biological role during cancerogenesis [1, 2]. The aforementioned analysis might be possible applying chip-based protein separation using Agilent 2100 Bioanalyzer. However it has not been defined if the above-mentioned device and methodology are suitable for analysis of FFPE extracts. To the best of our knowledge, there are only 2 publications [3, 4] concerning application of protein extracts for Agilent 2100 Bioanalyzer assay. However both of the aforementioned studies dealt with the analysis of cell lysates. Therefore in this paper, for the first time, we tested whether it is possible to separate proteins extracted from FFPE tissues with Agilent 2100 Bioanalyzer. It is widely accepted this kind of material may be problematic [5].

Material and Methods

FFPE tissues obtained from HER2-positive breast cancer patients as well as cell culture of fibroblasts were used.

Protein Extraction

Protein isolation from FFPE blocks was performed using Qproteome FFPE Tissue Kit (Qiagen, Germany) according to manufacturer recommendations. Briefly, FFPE sections were cut directly from the blocks, deparaffinized and rehydrated through a series of xylenes and ethanols. Next, the sections were incubated in Extraction Buffer EXB Plus (containing β-mercaptoethanol) at 100˚C and then at 80˚C to reverse formalin crosslinking and untangles the protein molecules. After centrifugation the supernatant was collected. Whole protein extracts from fibroblast cell culture, extracted using RIPA buffer, were kindly donated by PhD – Assoc. Prof Dorota Słonina. Total protein concentrations of all specimens were determined using Bradford assay (Bradford Reagent, Sigma-Aldrich, MO, USA). Samples were stored at -80˚C until further use.

On-chip electrophoresis by Agilent 2100 Bioanalyzer

For microchip electrophoresis samples were prepared using Agilent High Sensitivity Protein 250 Kit and analyzed by Agilent 2100 Bioanalyzer (both from Agilent Technologies, Germany). Agilent High Sensitivity Protein 250 Kit is designed for sizing and sensitive analysis of 10 to 250 kDa proteins and is based on the detection of fluorescently labeled proteins, that are separated electrophoretically on microfluidic chips [6].

The labeling protocol encompassed adding 1 µL of fluorescent dye to 10 µL of protein sample and incubation for 30 min on ice. Then the excess of dye was quenched by 1 µL of ethanolamine (incubation for 10 min on ice).

Next, immunoprecipitation was performed using Dynabeads Protein G (Life Technologies, Norway). Ten µL of labeled protein extracts (about 20 µg) was mixed with 100 µL of wash buffer (PBS with 0.1%Tween 20) and antibody detecting: (1) β-actin – which is constitutively expressed and used as a positive control (Clone AC-15, Sigma, MO, USA), and additionally (2) HER2 (we applied two antibodies against intracytoplasmic fragment of HER2 protein: Polyclonal Rabbit Anti-Human, Dako, Denmark or clone CB11, Leica Biosystems, UK) and the mixtures were incubated overnight at 4˚C with constant rotation. Next, 5 µL of Dynabeads was washed twice with wash buffer. Then they were diluted in 100 µl of wash buffer, mixed with protein-antibody complex and incubated for 2 hours at 4˚C with constant rotation. The supernatant was discarded and the complexes were washed 3 times with 200 µL of wash buffer. Extra care was taken during the last step to remove the remaining liquid completely. Finally, to elute all bound proteins, the beads were resuspended in 10 µL of water and 5 µL of sample buffer containing dithiotreitol. After heating at 95˚C for 5 min samples were centrifuged, beads were separated on the magnet and supernatant containing eluted protein (and antibody), was loaded directly onto the microchip according to manufacturer instructions. The ladder labeling and preparation for on-chip analysis was performed following manufacturer's recommendations.

Western Blot

About 50 µg of proteins extracted from FFPE were mixed with 6 x SDS loading buffer (containing bromophenol blue dye) and then heated at 95˚C for 10 min. Samples were loaded on 10% gel and SDS-PAGE electrophoresis was run at 150 V. Then, proteins were transferred onto Immobilon-P PVDF membrane (Millipore Corp., MA, USA) under constant current (250 mA) for 1 hour at 4˚C. The membrane was blocked with 10% non-fat milk in TBS-T (Tris Buffered Saline containing 0.1% of Tween 20) for 1 hour at room temperature (RT). Then, the following steps were performed: (1) membrane incubation with primary antibody against β-actin (Clone AC-15, 1:4000, Sigma, MO, USA) overnight at 4˚C, (2) wash with TBS-T for 1h at RT (buffer was changed every 15 min), (3) incubation with HRP-conjugated secondary antibody (1:10000, Santa Cruz Biotechnology, TX, USA) for 45 min at RT and (4) wash with TBS-T (as described above). All membrane incubation steps

were performed under constant shaking. The expression levels of β-actin were visualized using Amersham ECL Prime Western Blotting Detection Reagent (GE Healthcare, IL, USA) according to manufacturer instructions.

Immunohistochemistry (IHC)

Four-µm-thick formalin-fixed parafin-embedded sections of 2 tissues (case no 1 and 2) were mounted on SuperFrost Plus (Menzel-Gläser, Germany) slides. Then they were dewaxed (xylene), rehydrated (series of alcohols) and subjected to the following procedures: (a) antigen retrieval (Target Retrieval Solution – TRS, pH=6.1; 96˚C; 40 min; Dako, Denmark), (b) quenching the activity of endogenous peroxidases (0.3% H_2O_2 in 100% methanol; 30 min incubation; RT), (c) blocking of unspecific antibody binding (UltraVision Protein Block: 5 min; RT; ThermoScientific, CA, USA), (d) incubation with anti-CD34 antibody (clone QBEnd 10; dilution 1:50; overnight incubation at 4°C; Dako, Denmark), (e) incubation with BrightVision Detection System (30 min; RT; Immunologic, The Netherlands) and 3,3'-diaminobenzidine (DAB; Vector Laboratories, CA, USA). Eventually, slides were counterstained with Mayer's hematoxylin.

To detect the effect of Extraction Buffer and β-mercaptoethanol on antigen-antibody binding, anti-CD34 antibody was diluted in 3 different buffers: (1) Tris Buffered Saline with 0.1% Tween 20 (TBS-T, positive control), (2) TBS-T with β-mercaptoethanol and (3) TBS-T with Extraction Buffer from Qproteome FFPE Tissue Kit. The concentrations of Extraction Buffer and β-mercaptoethanol in antibody solution for IHC were calculated to be the same as in the immunoprecipitation step in the Bioanalyzer procedure.

Results and discussion

In this study we tried to detect β-actin and additionally truncated form of HER2 protein in extracts from FFPE using Agilent 2100 Bioanalyzer.

In extracts from FFPE, using the above-mentioned methodology, during preliminary experiments, we failed to detect neither β-actin (42 kDa) nor full length (185 kDa [1]) or truncated HER2 (95-100 kDa [1]), (Figure 1). In presented analysis probably only bands from heavy (around 75 kDa) and light (around 23 kDa) chains of antibodies were visible (Figure 1). The last observation suggested that Protein G (coating the surface of magnetic beads) is able to interact with antibody, so the antibody could be immunomagnetical-

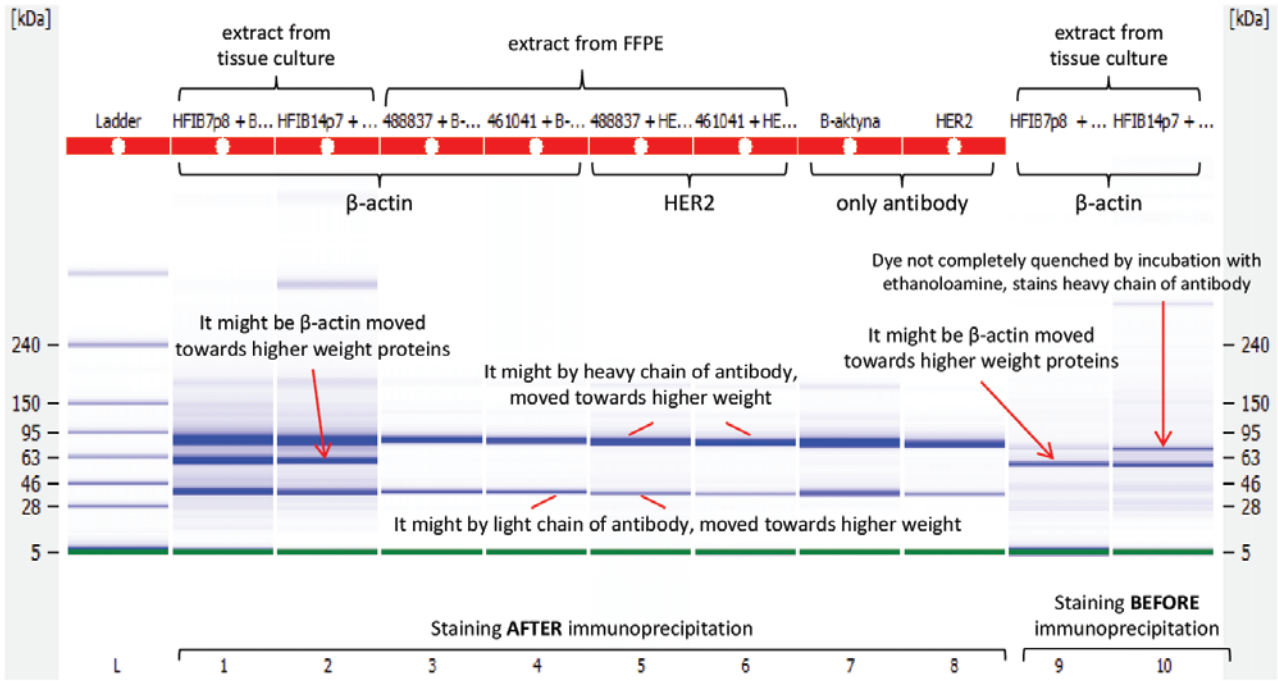

Figure 1. Results obtained using Agilent 2100 Bioanalyzer after immunomagnetic separation of HER2 and β-actin proteins from FFPE and tissue culture extracts.

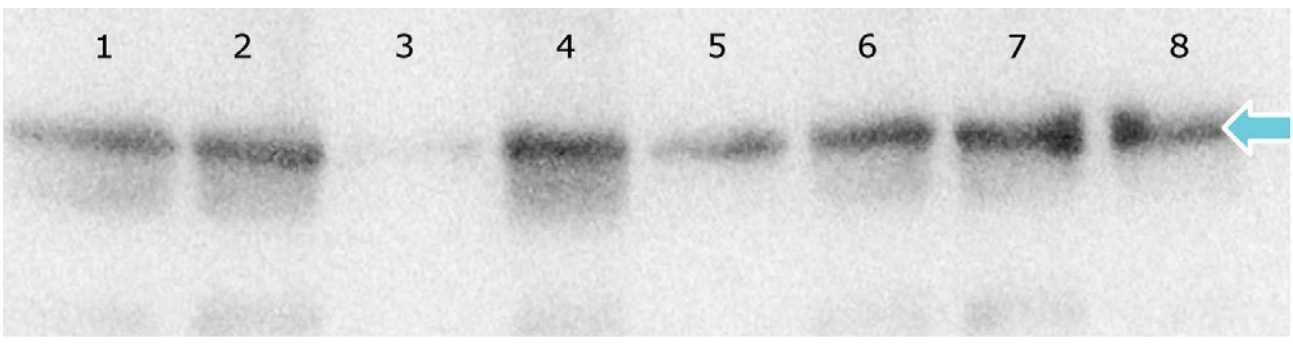

Figure 2. β-actin band (indicated with an arrow) visualized after Western Blot analysis of FFPE protein extracts (samples 1– 8, about 50 µg of protein per well).

ly separated and visualized after electrophoresis (high temperature incubation destroyed bounds between heavy and light antibody's chains, let us to observe 2 bands on the gel).

Although we were not able to detect β-actin band in FFPE extracts after immunomagnetic separation, in classic Western Blot analysis the band was visible (Figure 2). However, as depicted on Figure 2, despite the similar amount of the protein loaded to all wells (50µg) in sample no 3 and 5 β-actin band was barely visible. Moreover in all analyzed cases we observed strong signal from the background. Those facts might indicate poor quality of proteins extracted from FFPE samples. Moreover, when we used tissue culture protein lysates for Bioanalyzer analysis, β-actin band appeared (we think that it was moved towards high weight proteins – see Figure 1). The above-mentioned observations may suggest that the reason of failure in protein detection by Bioanalyzer after immunomagnetic separation is lack of binding between antibody and antigen (β-actin or HER2). Hence, we hypotesized that the interaction between antibody and antigen might be prevented by Extraction Buffer (from Qproteome FFPE Tissue Kit) or β-mercaptoethanol, which were both present in FFPE protein extracts.

We tested this conception during IHC procedure. We added Extraction Buffer and β-mercaptoethanol to properly diluted anti-CD34 (marker of endothelial cells, visualizing blood vessels) antibody, keeping the same concentrations of them as in the immunoprecipitation step (part of Bioanalyzer procedure). We selected CD34 for IHC, because from our previous experience, we know that CD34 staining is robust against small methodological variations and this marker is always present in sections from tumor tissue. The results are presented on Figure 3 and clearly shown that there was no staining if Extraction Buffer was added to antibody solution (Figure 3A and D), suggesting that the antigen-antibody binding was blocked in those cases.

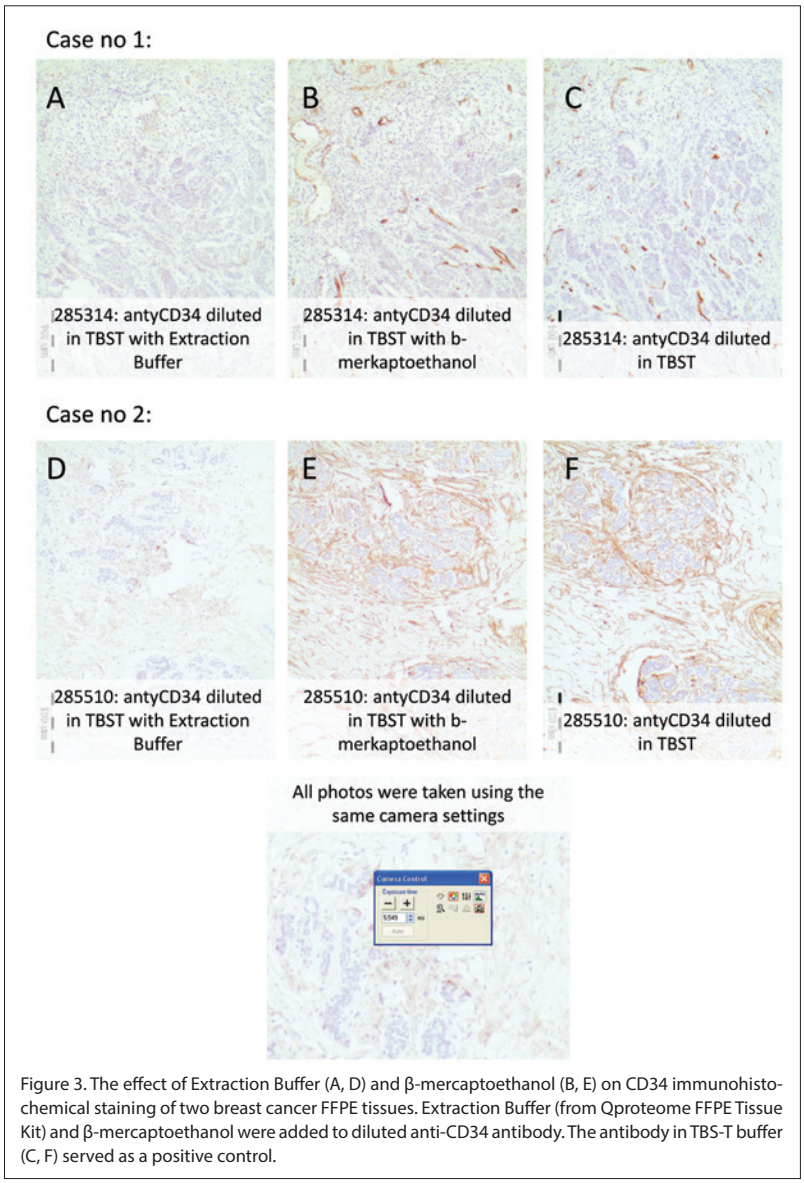

Figure 3. The effect of Extraction Buffer (A, D) and β-mercaptoethanol (B, E) on CD34 immunohistochemical staining of two breast cancer FFPE tissues. Extraction Buffer (from Qproteome FFPE Tissue Kit) and β-mercaptoethanol were added to diluted anti-CD34 antibody. The antibody in TBS-T buffer (C, F) served as a positive control.

To make sure that above-mentioned observations were not the effect of mistakes or omitting something important during on-chip electrophoresis and immunoprecipitation procedures, in the preliminary studies we tested many different possibilities and modifications of Agilent 2100 Bioanalyzer protocol, among others: (i) magnetic separation of different proteins and different antibodies used to the same protein (dedicated for IHC or Western Blot), (ii) different incubation times or samples/antibodies concentrations, (iii) labeling of whole protein extract or separated proteins – after forming the immunocomplexes to determine if labeling procedure prevented binding antibody to antigen.

None of tested options and protocol modifications allowed us to improve the obtained results and see protein signal after analysis with Bioanalyzer.

Concluding, in this paper for the first time we checked whether it is possible to separate proteins extracted from FFPE tissues using magnetic immunoprecipitation and Agilent 2100 Bioanalyzer. We found that Extraction Buffer (which is a component of Qproteome FFPE Tissue Kit) prevents an interaction between antibody and antigen so these extracts were not suitable for Bioanalyzer on-chip analysis.

Conclusion

Qproteome FFPE Tissue Kit is not suitable for protein analysis using Agilent 2100 Bioanalyzer, because obtained protein extracts contain the Extraction Buffer, which seems to prevent an interaction between antibody and antigen during immunoprecipitation procedure.

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