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Research Article

A database for inventory of proteoform profiles: "2DE-pattern"

The human proteome is composed of a diverse and heterogeneous range of gene products/proteoforms/protein species. Because of the growing amount of information about proteoforms generated by different methods, we need a convenient approach to make an inventory of the data. Here, we present a database of proteoforms that is based on information obtained by separation of proteoforms using 2DE followed by shotgun ESI–LC–MS/MS. The database's principles and structure are described. The database is called "2DE-pattern" as it contains multiple isoform-centric patterns of proteoforms separated according to 2DE principles. The database can be freely used at http://2de-pattern.pnpi.nrcki.ru.

Keywords:
2DE / database / pattern / protein species / proteoforms
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1 Introduction

Detailed information about all human proteins is a main target of the Human Proteome Organization (HUPO). Until now, the main efforts of the scientific community were focused on finding the proteins coded by the corresponding genes. This task is close to completion now as from the list of 20 199 predicted proteins only ≈2000 proteins remain as so-called "missing proteins" and attract the special attention of Chromosome-Centric Human Proteome Project (C-HPP) community. But the situation is much more complicated than that as proteins coded by the same gene can exist in different forms [1,2]. There are different processes generating the forms. Some of them use genetic variations at the mRNA level, such as alternative splicing, different promoters, and translation initiation sites. These protein forms are called "isoforms" [3,4]. Additionally, these isoforms can be further chemically modified by posttranslational modifications (PTMs). The final products are called protein species [1,5] or proteoforms [6,7]. Because of different combinations of these events, theoretically the human proteome could encompass billions of proteoforms [8–11]. Thus, from the same open reading frame (ORF) product, multiple proteoforms that fulfil different functions can exist. Accordingly, there is a need for a comprehensive inventory of all that variety. As proteomics goes deeper and deeper into the heterogeneity of proteins; the volume of information is growing very fast, so there is a high demand for convenient ways to use this information. Because of the huge amount of data generated, protein databases are a crucial part of proteomics. Indeed, a search of sequence databases is a first step in proteomics based on MS. Multiple databases have been developed and are available. The well-known databases are in the National Center for Biotechnology Information (NCBI; http://www.ncbi.nlm.nih.gov) and SIB Bioinformatics Resource Portal ExPASy, https://www.expasy.org/. Some of them are NeXtProt, UniProt, and SWISS-2DPAGE [12–14]. Usually, the databases are based on the specific method by which the data were generated. The separation method that is ideally suited for proteome analysis is 2DE. Because of the parameters that are used in this method, it provides a suitable basis for the development of a proteome database. Accordingly, multiple 2DE-based protein databases for different sample sets, including human samples, for which bottom-up MS is widely used, have been built [15]. However, it became evident that for the physicochemical characterization of proteoforms, top-down MS would be necessary. Therefore, a proteomics project, for which top-down MS was used, was initiated [16], and inside this project, a proteoform database (Proteoform Atlas, http://atlas.topdownproteomics.org) was organized [16,17]. Information about proteoforms can be also obtained by another approach—combination of 2DE with shotgun ESI–LC–MS/MS of proteins from the detected spots. Using this approach, it was shown that it is possible to detect multiple proteoforms even in a single protein spot

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Abbreviations: **ABC**, ammonium bicarbonate; **emPAI**, exponential modified form of protein abundance index; **FDR**, false discovery rate; **IAM**, iodoacetamide; **MALDI TOF**, matrix-assisted laser desorption/ionization; **PNPI**, Petersburg Nuclear Physics Institute; **PTM**, posttranslational modification; **SIB**, Swiss Institute of Bioinformatics

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[18–21]. It also became apparent that when using spot analysis after 2DE, perhaps a lot of information is lost. To solve this problem, we have developed a strategy of panoramic analysis (including so-called pixel-picking and semi-virtual approaches) using 2DE followed by shotgun ESI–LC–MS/MS [22–26]. Also, we reported the main technical aspects of developing an inventory of human proteoforms that would be visually attractive, clear, and easy to search [27,28]. In our view, this inventory could use the benefits of our methodology [27]. It should be noted that recent publications of other laboratories have confirmed fidelity of our approach [29]. As we generated a body of information about proteoforms using top-down proteomics (2DE separation followed by shotgun ESI–LC–MS/MS) that was conveniently organized in a graphical way, we decided to build a database, where that information could be kept and used by the scientific community. Accordingly, that database was constructed and it can be freely accessed at http://2de-pattern.pnpi.nrcki.ru. In the first draft of our database, there are only two samples—glioblastoma cells and lung embryonic fibroblasts [30]. More samples, such as normal and cancer cells (hepatoblastoma carcinoma (HepG2), HeLa, and HEK) and tissues (liver and blood plasma) will be incorporated later.

2 Materials and methods

2.1 Sample preparation

The cells (a primary line L of glial tumor origin, developed in the laboratory of cell biology, PNPI or LEH, human embryonic lung fibroblasts) were cultured in DMEM or RPMI-1640 media containing 5% fetal calf serum in 5% CO_2 at 37°C without antibiotics [30,31]. Samples were prepared as described previously [32]. Cells ($\approx 10^7$) containing 2 mg of protein were lysed in 100 μL of lysis buffer (7 M urea, 2 M thiourea, 4% CHAPS, 1% dithiothreitol (DTT), 2% ampholytes, pH 3–10, protease inhibitors mixture). The protein concentrations in the samples were determined by Bradford assays [33].

2.2 Two-dimensional gel electrophoresis

All procedures were performed as described previously [22–25]. In short, in case of a classical 2DE, it was applied 1000 μg of protein to 24 cm IPG nonlinear strip. After IEF, the strip was placed on the top of a 12% polyacrylamide gel and electrophoresed in a second direction under denaturing conditions [24,31]. The gel (1 mm × 25 × 20 cm) was stained with Coomassie Blue R350, scanned by ImageScanner III (GE Healthcare, Pittsburgh, PA, USA), and analyzed using ImageMaster 2D Platinum 7.0 (GE Healthcare).

In case of a sectional 2DE, 500 μg of protein was applied to 7 cm IPG linear strip. After second dimension, the gel (1 mm x 8 × 8 cm) was stained, scanned, analyzed by ImageMaster 2D Platinum 7.0, and divided into 96 sections with determined coordinates identified as 1–12 along the Mw dimension and A–H—along the pI dimension. All procedures were performed according to the protocol described previously [24]. Each section was treated with trypsin. Tryptic peptides were eluted from the gel with extraction solution (5% (v/v) ACN, 5% (v/v) formic acid) and dried in a vacuum centrifuge Speed Vac. Peptides were dissolved in 5% (v/v) formic acid.

In case of a semi-virtual 2DE [25], a first step was the same as in a classical 2DE (a 24 cm IPG nonlinear strip and 1 mg of protein). After IEF the strip was cut into 48 equal sections (5 mm), and each section was transferred to Eppendorf tube and treated according to trypsin digestion protocol. Samples were digested overnight at 37°C. Supernatants that possibly contained peptides that had diffused out of the gel slices were collected. Peptides were extracted additionally by 150 μL of 60% ACN, and 0.1% trifluoroacetic acid (TFA). Extracts were dried in Speed Vac, and reconstituted in 20 μL of 0.1% TFA, before being analyzed by MS.

2.3 MALDI–MS and ESI–LC–MS/MS analyses

All procedures were performed according to the protocol described previously [23,32,34]. MALDI–TOF-MS was performed using Microflex MALDI/TOF (Bruker Inc, Bremen, Germany). Tandem MS analysis was carried out on an Orbitrap Q-Exactive mass spectrometer (Thermo Scientific, Waltham, MA, USA) according to the protocols described previously [35].

2.4 Protein identification

Identification of the proteins was performed using Mascot "2.4.1" (Matrix Science, London, UK) by searching the UniProt/Swiss-Protein sequence database (October 2014, 20 196 total sequences). The following search parameters were used: trypsin—as the cutting enzyme, the mass tolerance for the monoisotopic peptide window was set to ±20 ppm, missed cleavages – 1. Cysteine carbamidomethyl was chosen as a fixed modification. A combination of three variable modifications (acetylation of lysine, acetylation of N-end, phosphorylation of serine/threonine or tyrosine, and oxidation of methionine) was used. For false discovery rate (FDR) assessment, a separate decoy database was generated from the protein sequence database. A false positive rate of 1% was allowed for protein identification. A minimum Mascot ion score of 30 was used for accepting peptide MS/MS spectra. Data were also searched, using the SearchGUI, an open-source graphical user interface [36]. Two unique peptides per protein were required for all protein identifications. Exponentially modified PAI (emPAI), the exponential form of protein abundance index (PAI) defined as the number of identified peptides divided by the number of theoretically observable tryptic peptides for each protein, was used to estimate protein abundance [37]. All additional information about the methods can be also reached through the front page

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of the database by clicking the corresponding links for protocols or articles.

2.5 Software used for database construction

All data were stored in a Mysql database (version 15.1) on Linux server. Scripts that process requests from the user interface were developed using Perl 5 (version 30). Website is based on webserver Apache / 2.4.39. Interactive user interface to database is implemented using HTML, CSS, JavaScript, and JQuery library. The queries to server programs are executed using AJAX technology.

3 Results and discussion

3.1 Overview of the database

Our main goal is to construct a proteoform database based on 2DE principles. That database was meant to provide a comprehensive and simple tool to store and share information about human proteoforms with the scientific community. As each proteoform is a unique molecule (polypeptide), a central point in our strategy was the usage of such specific polypeptide parameters as isoelectric point (pI) and molecular weight (Mw). The combination of those physicochemical parameters gives a convenient representation of each polypeptide. It is very felicitous that in the classical 2DE, separation is performed exactly according to those parameters. It is logical that the SWISS-2DPAGE database that based on these parameters was developed [12,13]. Because of the high popularity of 2DE, the SWISS-2DPAGE became a part of the federated 2DPAGE database [38]. Following, in many aspects, the organizational style of this database we constructed our proteoform database. Compared to the SWISS-2DPAGE database this database has some additions. To obtain information about proteoforms, we use three different approaches based on 2DE separation and identification by MS. The first approach is based on a classical 2DE, which is mainly performed according to rules used in the SWISS-2DPAGE and the federated 2DE databases [38]. The second one is a sectional 2DE, when a whole gel, not only selected spots, is analyzed by ESI–LC–MS/MS section by section. The third one is a semi-virtual 2DE, in which proteoforms are separated only by IEF according to their pI. Each approach allows one to produce a specific proteoform pattern for every isoform. All three approaches have limitations, but they are complementary to each other and allow us to obtain a better view of the combined proteoform profiles of isoforms.

3.2 Database content and search features

Our laboratories have expertise in proteomics based on the analysis of proteins and proteoforms using 2DE and MS (MALDI-TOF and ESI–LC–MS/MS) [27]. We are using different samples, and information about proteoform patterns obtained from experiments performed with those samples will be included in the database. This information was already discussed, peer reviewed, published, but additionally was processed according to the latest changes in the Uniprot database (December 2019: 20 367 reviewed human). Each entry in the database corresponds to one protein isoform and contains textual and graphical data. Every search starts from the "Entry page" (Fig. 1), where three different protein search options are offered: 1, UniProt accession number of the protein; 2, name of the protein; or 3, name of the gene. Additionally, on the "Entry page," users can find links to the methods (Protocols) and papers (References) that contain information that was included in the database. After the choice taken, it will be transition to the "Protein page" (Fig. 1), where the basic information about the protein is presented. The ID (identification) and the AC (accession number) are the same as the corresponding sequence entry in SWISS-PROT. The description line contains general information about the protein. The page also contains a subset of links to protein portals such as Proteoform Atlas, UniProt, and NeXtProt. Moreover, accession numbers and names of all known isoforms, according to how they are defined and included in the Uniprot database, are presented here. By clicking on the isoform accession number, the user is transferred to the page of this isoform ("Isoform page"; Fig. 1). Additionally, there is a link to the Table, where all detected isoforms for each sample is presented. Currently, we have data for 3498 isoforms of glioblastoma and for 4037 of lung fibroblasts. The "Isoform page" has links to three pages: "2DE page," "sectional 2DE page," or "semi-virtual 2DE page," where proteoform patterns that are produced by each of these approaches can be found (Fig. 1). In the "2DE page," a user can find a proteoform pattern of the isoform that is obtained by a classical 2DE (Fig. 2A). These data are organized based on similar principles as the SWISS-2DPAGE database and the way the federated 2DE database is built (http://world-2dpage.expasy.org/). In the 2DE map, spots, where different proteoforms of the same isoform were detected, are highlighted. Additionally, basic experimental information about the spot abundance and the isoform (isoform name, protein name, gene name, chromosome, theoretical pI, theoretical Mw, experimental pI, theoretical Mw, emPAI, and modifications) is shown. Minimal information obtained by mass spectrometric analysis is shown in the table. Additionally, by clicking on a spot, a user can extract information about all the isoforms that were detected in the spot. Furthermore, there is an option to check each chromosome for the detected proteoforms. At the time of this paper's submission, we identified for glioblastoma ~700 spots/isoforms, for lung fibroblasts, 149 spots/isoforms. A second type of data is presented in the "sectional 2DE page" [24] Therein, two images are presented, a whole image of a 2DE gel with its sections and a graph showing the distribution of specific proteoforms around these sections (Fig. 2B). A third type of a pattern can be found in the "semi-virtual 2DE page" (Fig. 2C). Inside the graph, proteoforms are represented proportionally (according to emPAI) by balls with different sizes. Moreover,

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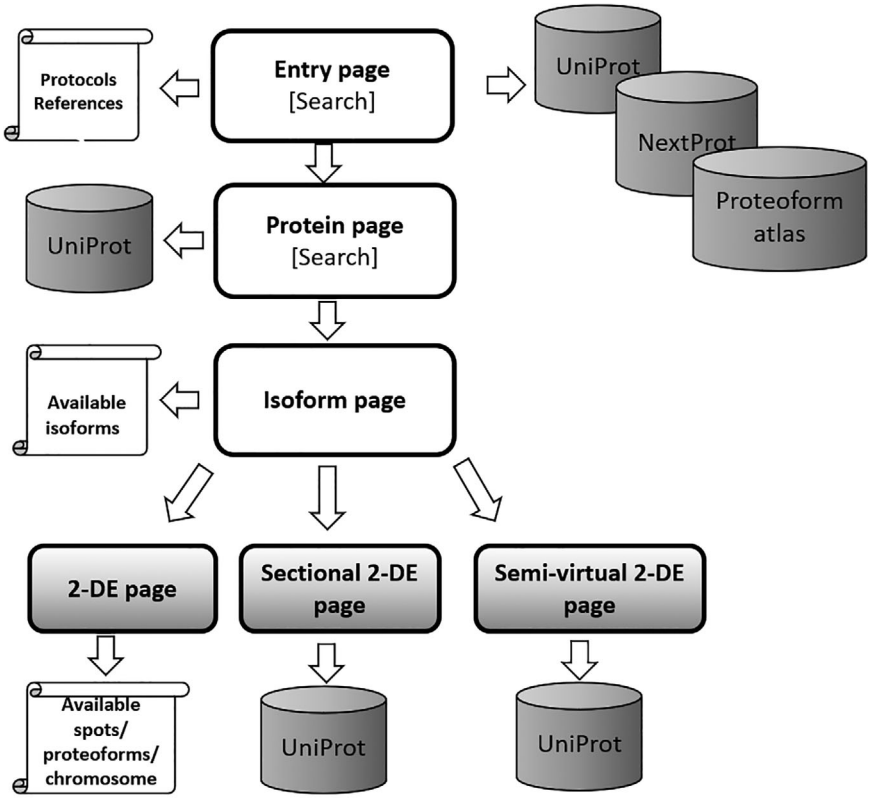

Figure 1. A flow chart of the proteoform database "2DE pattern." A user starts from the top, "Entry page," where a choice of search parameters can be found. According to the choice, a "Protein page" of the desired protein will be open. Beneath, a choice of isoform for this protein is available. From "Isoform page," a selection of three types of 2DE pattern for the isoform is available: "2DE page," "Sectional 2DE page," or "Semi-virtual 2DE page").

available information about the isoform and detected proteoforms is presented in the tables.

All the patterns obtained have advantages and disadvantages. The pattern obtained by a classical 2DE has high resolution but is limitted to the number of proteoforms as they are detected only in the stained spots. The pattern obtained by a sectional 2DE has low resolution (several proteoforms can be seen as a single one) but covering a whole gel and captures proteoforms that are present in the gel but not visible by staining. Peptides derived from those proteoforms are able to be detected, but the proteoforms would not normally be excised for analysis, because they are not visible by staining. The pattern obtained by a semi-virtual 2DE has high enough resolution in the pH direction but has no information about the real Mw of the proteoforms. The examples are presented in Fig. 2. Here, the graphical images representing distribution of proteoforms of adenine phosphoribosyl transferase (APT, isoform 1, identifier: P07741-1) and hypoxanthine-guanine phosphoribosyl transferase (HPRT, isoform 1, identifier: P00492-1) using three different approaches are shown (Fig. 2A,B,C). If we combine information about all these patterns together it allows us to come closer to a so-called "ideal"proteoform pattern (Fig. 2D). Such a pattern can be also produced by Western blotting if the antibodies used are specific for all the proteoforms in the pattern [28].

Importantly, from these pages, a user can go over to Uniprot or Proteoform Atlas. In UniProt, available information about PTMs of the isoform can be found. In Proteoform Atlas, it is possible, in some cases, to find proteoforms that have already been detected by top-down MS. Right now for APT (P07741-1) and HPRT (P00492-1) Uniprot and Proteoform Atlas have information about acetylated and phosphorylated proteoforms. Accordingly, we also expect to detect these PTMs (Fig. 2). Our database has only minimal information about PTMs that could be responsible for the variety of observed proteoforms (patterns). The database needs more information about PTMs, which we are trying to extract from our crude MS data. We have plans to include this information into the database gradually, after more detailed MS data analysis. Moreover, the website currently presents information only about glioblastoma and fibroblast (LEH) proteins. More information using other samples (proteins from HepG2, HEK, HeLa, liver, and plasma) are available and will be included into the database soon.

4 Concluding remarks

This work describes the database "2DE-pattern" that was developed for storing and sharing information about patterns or profiles of human proteoforms. These patterns were generated by separation of proteoforms, their identification by MS, and the construction of multiple graphical images, where proteoforms of the same isoform are presented. Separation was performed according to pI/Mw parameters using three

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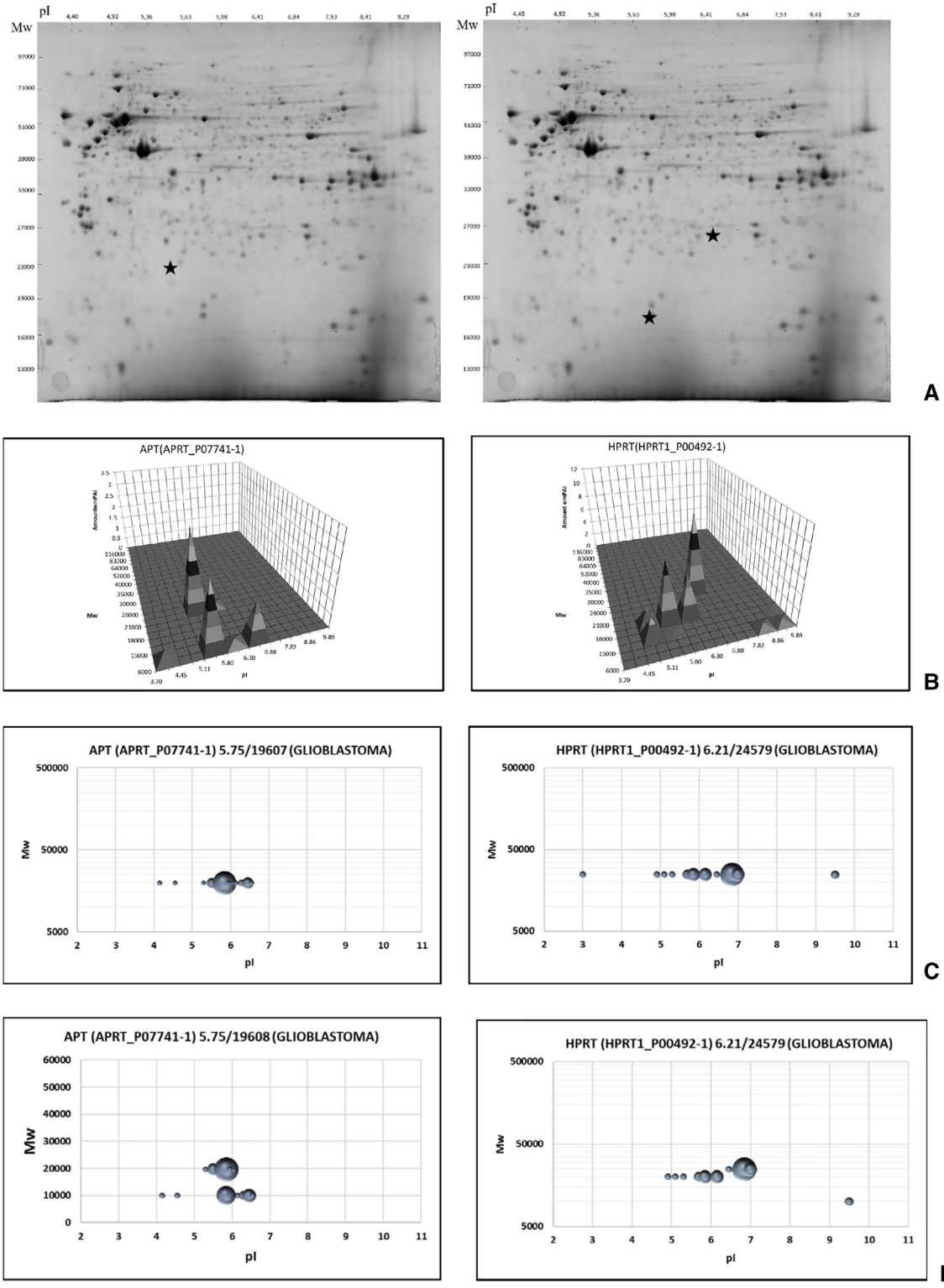

Figure 2. The examples (for glioblastoma) of the proteoform patterns for adenine phosphoribosyl transferase, APT (P07741-1), (left), and for hypoxanthine-guanine phosphoribosyl transferase, HPRT (P00492-1) (right). **(A) A classical 2DE**. The spots, where proteoforms were detected are marked by stars. **(B) A sectional 2DE.** The distribution of the proteoforms along the sections (proteoforms pattern) is shown. **(C) A semi-virtual 2DE**. **(D)** A combined graphical image representing data shown in **(B)** and **(C).** Here, many proteoforms got their real Mw (according to **(B)** patterns).

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variations of a classical 2DE. Accordingly, there can be up to three different proteoform profiles for each protein isoform entry in the database. All those patterns are complementary to each other and allow us to come closer to the complete set of proteoforms. In some cases, information about the PTMs of these proteoforms that was obtained from MS data is available. Additionally, based on pI/Mw parameters, it is possible to have a hint as to whether a proteoform has any PTMs or not. It is important to point out that, usually, in the most abundant proteoforms, the theoretical and experimental pI/Mw parameters are very similar indicating that those proteoforms are most likely not modified. All types of a shift (left, right, up, and down) are the results of different types of PTM. Therefore, depending on the shift direction, we can propose what type of PTM has occurred. More detailed data can be also found by clicking the links to the UniProt database. Using the available information, the user will have a chance to construct a more detailed image of the proteoform pattern. We can expect that ideal situation only in the future, as right now, Proteoform Atlas has data of only about 9706 proteoforms detected from all human cells. According to the modest evaluations, just a single human cell has at least 70 000 proteoforms, and a human proteome could encompass billions of proteoforms [8–11,22].

The database is freely available at http://2de-pattern.pnpi.nrcki.ru.

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The authors have declared no conflict of interest.

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