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APPLICATION OF PREPARATIVE ELECTROPHORESIS FOR CLINICAL PROTEOMICS IN URINE: IS IT FEASIBLE?

PRIMENA PREPARATIVNE ELEKTROFOREZE U KLINIČKOJ PROTEOMICI URINA: JE LI TO IZVODLJIVO?

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Summary: Urine samples are easily attainable which makes them ideal substrates for biomarker research. Various techniques have been employed to unravel the urine proteome and identify disease biomarkers. Even though the presence of high abundance proteins in urine is not so pronounced as in the case of plasma, the presence of proteolytic products, many of which at low abundance, along with numerous frequently random chemical modifications, makes the analysis of urinary proteins challenging. To facilitate the detection of low abundance urinary proteins, in the study presented herein we applied two different electrophoretic techniques, preparative Lithium Dodecyl Sulfate (LDS)-PAGE in combination with 2-DE for urinary protein separation and enrichment. Our results indicate the effectiveness of this approach for the enrichment of low abundance and low molecular weight proteins and peptides in urine, and contribute towards the establishment of a urinary proteomic database. The application of this technique as a biomarker discovery tool faces several challenges: these include down-scaling of the technique, possible recompensation for the consequent expected decrease in protein resolution, by optimizing steps of the experimental workflow as well as getting a good understanding of the technical variability of the technique. Under these conditions, preparative electrophoresis can become an effective tool for clinical proteomics applications.

Keywords: preparative electrophoresis, 2DE, urine, clinical proteomics, proteome mining

Kratak sadržaj: Uzorke urina je lako uzeti i oni su stoga idealan materijal za istraživanje biomarkera. Za ispitivanje proteoma urina i identifikaciju biomarkera oboljenja koriste se razne tehnike. Iako prisustvo proteina u urinu nije takao izraženo kao u plazmi, prisustvo proteolitičkih proizvoda, od kojih se mnogi nalaze u maloj količini, uz brojne, često nasumične hemijske modifikacije, predstavlja izazov za analizu proteina u urinu. Kako bi se olakšala detekcija retkih proteina u urinu, u ovoj studiji primenjene su dve različite elektroforetske tehnike, preparativna LDS-PAGE u kombinaciji sa 2-DE, u cilju separacije i obogaćivanja urinarnih proteina. Naši rezultati ukazuju na efikasnost takvog pristupa za obogaćivanje retkih proteina i peptida male molekularne mase u urinu, i doprinosi formiranju baze podataka o proteomima u urinu. Postoji nekoliko izazova za primenu te tehnike kao sredstva za otkrivanje biomarkera: smanjenje obimnosti tehnike, moguća rekompenzacija za posledični očekivani pad rezolucije proteina, usavršavanjem koraka u eksperimentalnom radnom postupku, kao i sticanje detaljnog uvida u tehničku varijabilnost tehnike. Pod tim uslovima, preparativna elektroforeza može postati efikasno sredstvo za aplikacije u kliničkoj proteomici.

Ključne reči: preparativna elektroforeza, 2DE, urin, klinička proteomika, analiza proteoma

Introduction

Proteomic technology as a combination of biochemical procedures with high throughput bioanalytical techniques has proven to be an invaluable tool for biology and biomedical research, especially during the last decade. Thousands of proteins of any kind of biological material – cell lines, tissues or body fluids – can nowadays be analyzed routinely generating vast numbers of data, related to protein expression.

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Human body fluids have been a rather significant part of proteomic research. Amongst them urine represents the most easily attainable and consequently one of the most common samples in clinical analysis and diagnostics (1). The advantages of using urine for clinical proteomic applications have been recently reviewed (2, 3).

However, it should also be noted that urine analysis presents difficulties: urine proteome composition changes as a result of the hydration status, diet, time of collection etc. It is well known in analytical chemistry that the nature of the sample itself along with sampling procedures, handling and storage contribute at least by 40% to the overall precision in analyte determination. Having this in mind it is not difficult to foresee the problems emerging in proteomic analysis of urine, especially for full proteome and biomarker discovery studies. The European Kidney and Urine Proteomics Consortium (www.eurokup.org) has recently been established with the objective to address some of these problems, establish standardized procedures in urine proteome collection and analysis and increase inter-lab reproducibility and data comparability (4).

Preparative electrophoresis provides fractionation of proteins based on their size (5). As shown in *Figure 1*, proteins elute from the acrylamide gel into separate fractions corresponding to different mass ranges. These fractions may be subsequently analyzed for their content using electrophoretic and/or chromatographic approaches in combination with mass spectrometry.

We have employed preparative electrophoresis in combination with 2DE for the analysis of the urine proteome (6). Our results indicate this is an efficient approach for proteome mining. To employ the technique in biomarker discovery studies several issues have to be resolved including down-scaling, optimizing individual experimental steps of the workflow and minimizing technical variability.

Methods

Preparative electrophoresis of urine sample

Urine collection from healthy volunteers and preparative separation of urinary proteins were performed as previously described (6). In brief, a volume containing 100 mg (or 4 in the case of clinical samples) of urine proteins is diluted with 4×lithium-dodecyl sulfate (LDS)-PAGE sample buffer to a final concentration of 1× buffer (50 mmol/L Tris, 10% glycerol, 2% LDS, 5% β-mercaptoethanol, 0.5% bromophenol blue, pH 6.8). Sample is applied on the prep-cell device (Biorad) and is then separated through a 4% stacking and an 11% separating polyacrylamide gel by electrophoresis at 40 mA current at 4 °C. Eluted proteins are trapped on a 3500Da molecular weight cut-off filter and collected by the continuous flow of elution buffer (25 mmol/L Tris, glycine 0.198 mol/L, 0.1% CHAPS, pH 8) with the help of a peristaltic pump. Fractions are collected using an automated fraction collector and stored at 4 °C until further use. An initial evaluation of the protein content of the fractions is performed by 1D SDS gel electrophoresis. A protein concentration and desalting step usually involving ultrafiltration is necessary prior to IEF.

2DE analysis of human urinary proteins

Two-dimensional separation of the prep cell fractions of urinary proteins is conducted as previously described (6). In brief, a sample volume containing at least 200 μg of proteins is diluted with IEF sample buffer (7 mol/L urea, 2 mol/L thiourea, 50 mmol/L Tris, 2% CHAPS, 0.4% DTE, 0.1% bromophenol blue, pH 7.5) to a final volume of 400 μL. The sample is then applied on IEF cell focusing trays and strip rehydration takes place actively at 50 V and at 20 °C overnight (o/n). After strip rehydration and the simultaneous sample loading, IEF follows for about 100 000 Vh (250 V for half hour, linear gradient 0 to 5000 V in 12 hours, constant at 5000 V for 16 hours and final step at 500 V for conservation). Strips are then incubated for 15 min at room temperature with equilibration solution I (6 mol/L urea, 50 mmol/L Tris, 30% glycerol, 2.0% SDS, 30 mmol/L DTE) followed by a 15 min incubation with equilibration solution II (same composition as I but with 230 mmol/L Iodoacetamide instead of DTE) for reduction and alkylation of the protein spots. Strips are then applied on the top of polyacrylamide gels, by the use of 0.5% agarose in TGS buffer and SDS-PAGE run for 4 hours approximately at 15 Watt per gel. 2-DE gels are stained with colloidal Coomassie blue or silver based techniques (7, 8). Following this procedure gels are scanned, spots are detected and excised manually or automatically. Trypsin digestion is performed as previously described (6).

Protein identification

Tryptic digests are extracted by the addition of 10 μL extraction solution (50% acetonitrile, 0.1% v/v TFA) and 15 min incubation at room temperature. Peptide mixture (1.5 μL) is applied on an anchor chip MALDI plate with 1 μL matrix solution (65% ethanol, 32% acetonitrile, 0.03% TFA, 0.025% α-cyano-4-hydroxycinnamicacid) containing the peptides des-Arg-bradykinin (Sigma, 904.4681 Da) and adrenocorticotropic hormone fragment 18–39 (Sigma, 2465.1989 Da) as internal standards. Samples are analyzed in a MALDI TOF mass spectrometer (Ultraflex, Bruker Daltonics, Bremen, Germany). The peak list is created with Flexanalysis v2.2 software by Bruker. Smoothing is applied with Savitzky-Golay algorithm

(width 0.2 m/z, cycle number 1). Signal to noise is calculated by SNAP algorithm and a threshold ratio of 2.5 is allowed. Peptide matching and protein searches are performed automatically with Mascot Server 2 (Matrix Science) with the following parameters: monoisotopic masses, 1 miscleavage site, carbamidomethylation as fixed modification and oxidation as variable and a mass tolerance of 25 ppm. In addition, as applicable, lift MS-MS measurements are performed to increase resolution and peptide identification.

Results

Preparative electrophoresis provides highly efficient separation of proteins based on size (*Figure 1*). In our initial application of the technique (6), 100 mg of urinary proteins were analyzed by LDS-prep cell electrophoresis. A total of 144 fractions, 5 ml each, were collected. As expected, early fractions contained low MW proteins and peptides with continuous increase in MW with fraction number (*Figure 2*). A total of 1898 spots were detected in the 2DE gels from the various early fractions and subsequently analyzed by MALDI-MS. Using very stringent criteria for protein search, 778 proteins were identified, corresponding to 141 different gene products. In comparison to the unfractionated starting material, a more than 3-fold increase in the number of identified gene products was achieved. Interestingly, a marked increase in the number of the low molecular mass proteins and peptides was observed (6).

To employ prep-2DE for comparative studies in clinical proteomics applications down-scaling as well as an evaluation of the repeatability of the technique is required. Since, however, down-scaling inevitably results in a decrease in protein resolution, optimization of the various steps of the experimental procedure was performed, including the running conditions of preparative electrophoresis, fraction desalting prior to 2DE and electrophoretic separation and detection systems.

Three to four mg of urinary proteins was separated by the use of the mini-prepcell electrophoresis system. Preparative electrophoresis was conducted on 11% acrylamide gel using buffers containing LDS-Chaps, as described above (Methods and 6), or including ammonium acetate and/or octyl-β-glucoside (OPG). The latter were tested as a means to avoid subsequent desalting experimental steps while

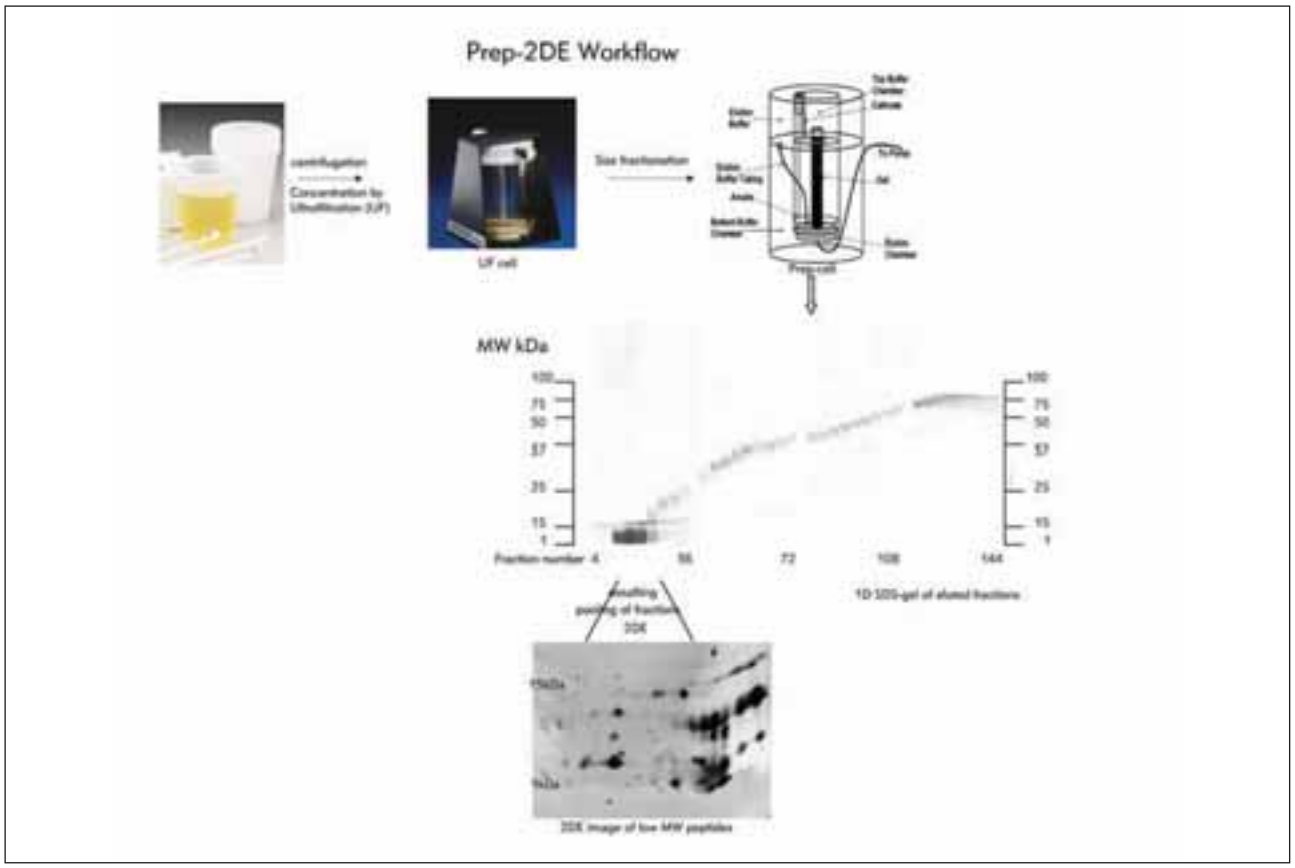

Figure 1 Experimental workflow in preparative electrophoresis: Urine samples are concentrated via ultrafiltration followed by protein separation according to size by preparative electrophoresis. Fractions are analyzed for their content by 1D and subsequent 2DE analysis.

maintaining compatibility with 2DE. However, the protein resolution in these cases was found inferior compared to LDS-Chaps which were therefore selected for use in subsequent analysis (data not shown).

Under these conditions 144 fractions of urinary proteins were collected. These fractions were combined into 5 different pools according to the protein mass, as follows: MW<15 kDa, 15<MW<25 kDa, 25<MW<37 kDa, 37<MW<50 kDa and MW>50 kDa.

For fraction concentration and desalting prior to their subsequent analysis by 2DE various techniques were tested including ultrafiltration, dialysis, TCA precipitation and protein purification with a commercial kit (ReadyPrep 2-D Cleanup Kit, Bio-Rad). Our results indicated dialysis as the most efficient desalting technique for low molecular weight peptides and ultrafiltration for the middle and higher molecular weight fractions.

Protein separation was conducted on acrylamide gels of optimal acrylamide concentration for the different mass ranges: 15% for MW<15 kDa, 11% for 15<MW<25 kDa and 25<MW<37 kDa, 8% for 37<MW<50 kDa and MW>50 kDa.

Preparative electrophoresis is a cumbersome experimental process involving multiple steps of analysis. Thereby, application of this system in the comparative analysis of clinical samples further requires the evaluation of its repeatability. From triplicate replications of the technique, an average matching rate of 80% of the received 2DE images from the respective fractions was determined.

Discussion

Preparative electrophoresis has already been used successfully for the enrichment of proteins in biological samples and has known pros and cons (5). In brief, it is labor intensive and not of high throughput capability. On the other hand, it is not as costly as liquid chromatography and it does not demand extensive method development studies. The size exclusion phenomenon which is the basis of PAGE serves as an ideal separation means prior to 2-DE. Proteins are eluted according to their molecular weight enabling proteomic analysis in specific molecular weight ranges. The subsequent use of large pH range strips (3–10) enables the detailed and high resolution separation of proteins within each of

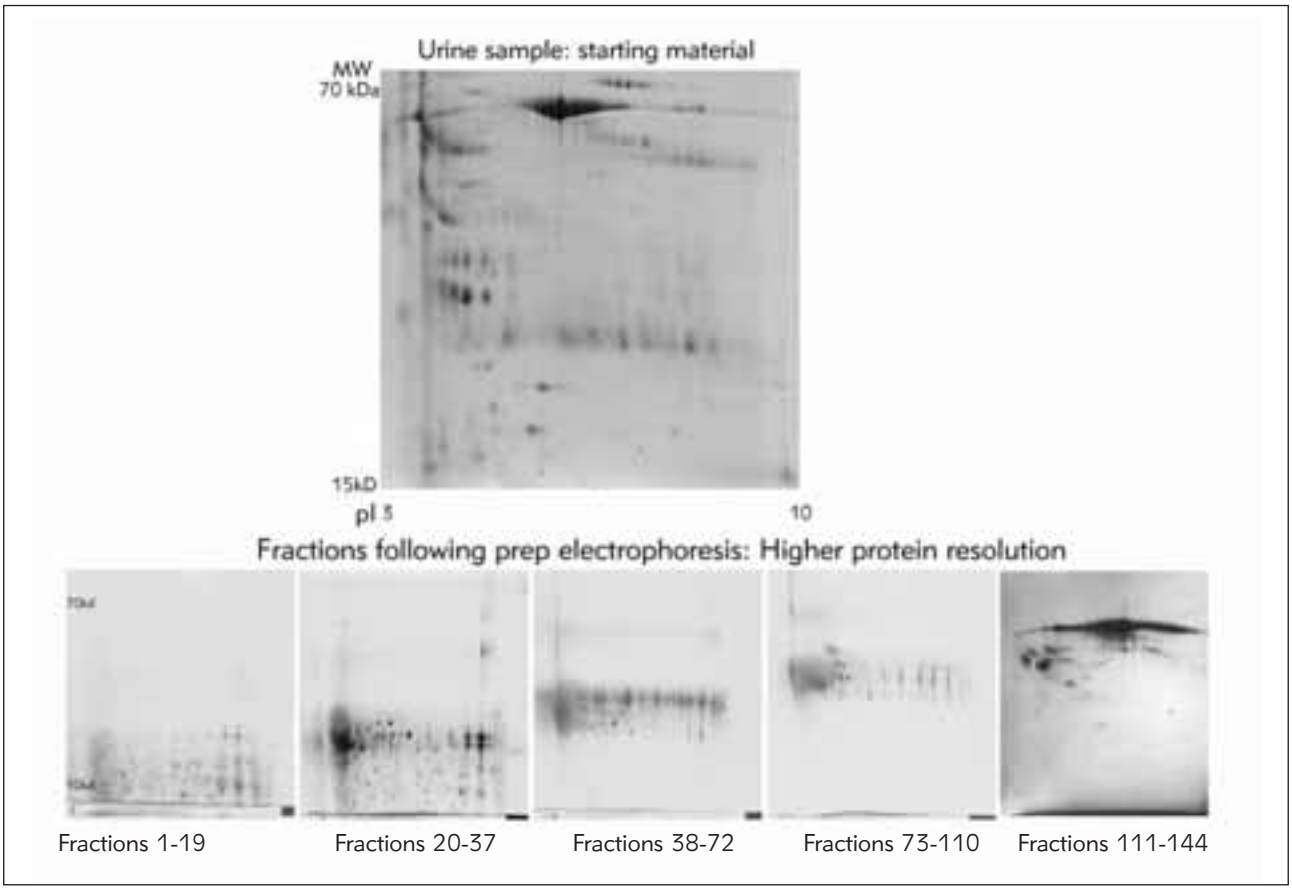

Figure 2 Increase in protein resolution, particularly in the low MW peptides following preparative electrophoresis. Top: 2DE image of the unfractionated starting urine; bottom: 2DE images of the protein fractions

these different molecular weight ranges. Therefore, preparative PAGE in combination with broad pI 2-DE separation may serve as an ideal means for the construction of proteomic maps, the enrichment of low abundance proteins and the detection of low MW proteins and peptides.

Comparison of the list of identified proteins following prep cell analysis with already published urinary protein databases showed similarity in the detection of abundant proteins (mainly deriving from plasma such as albumin, antibodies, etc.) (6, 9–11). Lower abundance and low molecular weight proteins, however, presented great variability. This can be attributed to the different sampling as well as sample preparation procedures employed in the various studies. In addition, the preparative electrophoresis methodology described appears to have enriched the urinary proteomic database for lower abundance proteins. Several enzymes of signal transduction pathways, metabolism and transport-carrier proteins were detected (6).

These results collectively support the view that the methodology may be well employed as a proteome mining approach. To make it applicable for the comparative analysis of clinical samples and the identification of potential disease biomarkers, several issues had to be resolved:

a) Down-scale the technique: collection of liters of urine from patients, even though not impossible, is quite difficult to be organized from the clinical point of view.

b) The procedure is quite cumbersome, involving multiple experimental steps. For this reason, a thorough evaluation of the technical repeatability of the approach has to be performed prior to any comparative studies. The matching rates received in our analysis may be improved by the combination of the process with protein labelling approaches (Difference Gel Electrophoresis). This is an approach currently followed in our laboratory.

c) Down-scaling inevitably results in a decrease in protein resolution. Even though this effect has been recompensated up to some extent by optimization of the various steps of the experimental workflow, still the effect is evident when employing colorimetric techniques for protein detection. An avenue for improvement in this case is the combination of the approach with fluorescent-based detection techniques.

In short, preparative electrophoresis is a very efficient tool for protein fractionation based on size. In combination with 2DE, it is an excellent tool for proteome mining; in addition, due to its efficiency in protein separation, it is a highly effective method for targeted proteomic applications involving the isolation and further study of selected proteins. Its employment in biomarker discovery is also feasible; nevertheless, caution is required during data interpretation in potential variability introduced for technical reasons. In general, as in every biomarker discovery approach using proteomics tools, a good understanding of the advantages and limitations of the employed technique is needed (12). Under these conditions, reliability of the analysis is maximized with a net result, the increase in the number of the identified potentially useful clinical biomarker candidates.

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References

1. Thongboonkerd V. Recent progress in urinary proteomics. Proteomics Clin Appl 2007; 1: 780–91.
2. Fliser D, Novak J, Thongboonkerd V, Argilés A, Jankowski V, Girolami MA, Jankowski J, Mischak H. Advances in urinary proteome analysis and biomarker discovery. J Am Soc Nephrol 2007; 18 (4): 1057–71.
3. Decramer S, Gonzalez de Peredo A, Breuil B, Mischak H, Monsarrat B, Bascands JL, Schanstra JP. Urine in clinical proteomics. Mol Cell Proteomics 2008; 7 (10): 1850–62.
4. Vlahou A, Schanstra J, Frokiaer J, El NM, Spasovski G, Mischak H, Domon B, Allmaier G, Bongcam-Rudloff E, Attwood T. Establishment of a European Network for Urine and Kidney Proteomics. J Proteomics 2008; 71: 490–2.
5. Fountoulakis M, Juranville J. Enrichment of low-abundance brain proteins by preparative electrophoresis. Anal Biochem 2003; 313: 267–82.
6. Zerefos PG, Vougas K, Dimitraki P, Kossida S, Petrolekas A, Stravodimos K, Giannopoulos A, Fountoulakis M, Vlahou A. Characterization of the human urinary proteome by preparative electrophoresis in combination with 2-DE. Proteomics 2006; 6: 4346–55.
7. Candiano G, Bruschi M, Musante L, Santucci L, Ghiggeri GM, Carnemolla B, Orecchia P, Zardi L, Righetti PG. Blue silver: a very sensitive colloidal Coomassie G-250 staining for proteome analysis. Electrophoresis 2004; 25 (9): 1327–33.
8. Chevallet M, Luche S, Rabilloud T. Silver staining of proteins in polyacrylamide gels. Nat Protoc 2006; 1: 1852–58.
9. Oh J, Pyo JH, Jo EH, Hwang SI, Kang SC, Jung JH, Park EK, Kim SY, Choi JY, Lim J. Establishment of a near-standard two-dimensional human urine proteomic map. Proteomics 2004; 4 (11): 3485–97.
10. Pieper R, Gatlin CL, McGrath AM, Makusky AJ, Mondal

M, Seonarain M, Field E, Schatz CR, Estock MA, Ahmed N, Anderson NG, Steiner S. Characterization of the human urinary proteome: a method for high-resolution display of urinary proteins on two-dimensional electrophoresis gels with a yield of nearly 1400 distinct protein spots. Proteomics 2004; 4 (4): 1159–74.

11. Adachi J, Kumar C, Zhang Y, Olsen JV, Mann M. The human urinary proteome contains more than 1500 proteins, including a large proportion of membrane proteins. Genome Biol 2006; 7 (9): R80.

12. Good DM, Thongboonkerd V, Novak J, Bascands JL, Schanstra JP, Coon JJ, Dominiczak A, Mischak H. Body fluid proteomics for biomarker discovery: lessons from the past hold the key to success in the future. J Proteome Res 2007; 6: 4549–55.

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