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Comparison of Different Protein Concentration Strategies by Two-Dimensional Gel Electrophoresis of Human Serum Proteomics

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In the research of serum proteomics, an appropriate sample preparation is essential for obtaining good results in two dimensional electrophoresis (2-DE). The concentration steps are necessary to sample preparation and several other processes. Our goal is to find the most efficient method that minimally impacted on serum proteins for a serum proteomics. We compared three strategies of concentration including ReadyPrep 2-D cleanup kit, freeze-drying and ultrafiltration using crude serum samples and high abundant proteins depleted serum samples. By comparing the different characteristics of the operation process, the recovery effect and the compatibility with 2-DE, it is found that the freeze-drying and ultrafiltration performed an efficient sample concentration of serum proteins. And all of these three concentration strategies delivered a satisfied compatibility with 2-DE technique of serum proteomics. Less manual operations involved in concentration procedures was expected to provide a more reliable result of serum proteomics.

Key Words: Serum, Biomarker, Two-dimensional gel electrophrosis, Freeze-drying, Ultrafiltration.

INTRODUCTION

A large number of studies for human diseases have been using proteome-based high throughout techniques for serum proteomics[1,2]. So far, there are several commercial kits available for depletion of 1 to 12 kinds of high abundant protein from serum[3-5]. In order to meet the demand of some experiments, crude serum samples usually need to be pretreated to obtain the complexity of serum components. One of the crucial problems to avoid some biases in research of serum proteomics is the rational and reliable collection and pretreatment of crude serum samples[6].

During the experiment of 2-DE, for instance, the crude sera may be diluted to a suitable low concentration when depleting high abundance proteins using some depletion strategies with their protocols. However, the treated proteins may need to be concentrated to a relatively high concentration, which is convenient for the consequent studies. Usually, there are several kinds of methods for concentrating, such

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as trichloroacetic acid (TCA), acetone, chloroform/methanol and ammonium sulfate[7]. Moreover there are many recent commercial kits and devices with convenient, reproductive and high effective strategies for protein concentration.

We conducted human serum and compared the most widely used concentration methods by two-dimensional gel electrophoresis of human serum proteomics. In this study, ReadyPrep 2-D cleanup kit, freeze-drying and ultrafiltration were used in order to select the most reliable one for a high throughput sample analysis.

EXPERIMENTAL

The IEF system (IPGphore), Immobiline Dry Strips (17 cm, pH 3-10 nonline), CHAPS, DTT, *Tris*, glycine, methanol, urea, acrylamide and *bis* were purchased from Bio-Rad (Hercules, CA, USA). SDS, iodoacetamide and TEMED were obtained from Sigma-Aldrich (St.Louis, MO, USA). Proteo Extract Albumin/IgG Removal Kit was from Merck (Merck Darmstadt, Germany).The freeze-drying was carried out in VirTis BT 3.3 EL benchtop freeze dryer (The VirTis Cpmpany, Inc. USA), Centrifuge 5810R was obtained from Eppendorf (Eppendorf AG, Germany).

Serum samples collection and treatment: Pooled human serum was provided from 10 healthy Chinese people. 4 mL blood sample was collected pre-operatively in glass tubes without additive (4 mL BD VacutainerTM No Additive, BD, Franklin Lakes, NJ) and allowed to clot at room temperature for 40 min. Serum was separated by centrifugation at 2000 rpm for 15 min. Divided 200 μL aliquots of serum were taken and stored at -80 °C until ready for use. Besides the crude serum samples, a treated sample depleted high abundant protein of serum by Proteo Extract/Albumin/IgG Removal Kit (purchased by Merck Darmstadt, Germany) was used to the comparison of different concentration methods. The depletion procedures were performed at room temperature according to manufacturer's instructions and then were immediately frozen to -80 ºC. The time from collection to frozen storage was more than 1 h.

Concentration methods of serum proteins

Pre-solution of crude serum: The crude serum (50 μL) was diluted to 5000 μL with protein solubilization buffer (PSB). Following this step, the diluted serum and the high abundance depletion serum were divided into 200 μL aliquots and then they were stored separately at -80 ºC and were ready for use.

Precipitation by ReadyPrep 2-D cleanup kit: 1000 μL diluted serum and 1000 μL high abundance depletion serum were treated by ReadyPrep 2-D cleanup kit (Bio-Rad, Hercules, CA, USA). All precipitation procedures were performed at room temperature according to manufacturer's instructions. Precipitated samples were resuspended by PSB to about 150 μL.

Freeze-drying: 1000 μL diluted serum and 1000 μL high abundance depletion serum were placed in a freezer at -80 ºC for at least 45 min and then were dried under vacuum for 16 h. Lyophilized samples were reconstituted to about 150 μL of PSB.

Ultrafiltration: 1000 μL diluted serum and 1000 μL high abundance depletion serum were placed into an Amicon Ultra-15 5k device (Millipore Co.) and centrifuged at 3200 g 20 ºC for 1 h until the volume of the concentration reached about 150 μL. Then the concentration of the treated samples was estimated immediately.

Protein assays: Total protein content of crude and depleted serum samples were determined by the DC Protein Assay (Bio-Rad) according to the manufacturer's instructions with bovine serum albumin (BSA) as standards (Pierce, Rockford, IL, USA).

1-DE and gel staining: Treated and crude serum protein were analyzed on a SDS-PAGE 4-12 % *Bis-Tris* gel (1.0 mm × 10 well) (Bio-Rad, Hercules, CA) with loading amount of 20 μg of protein for each fraction. These proteins were resolved with the SDS-PAGE running buffer in a Novex Mini-Cell system (Invitrogen) at 200 volts (Powerpac power supply, Bio-Rad, Hercules, CA). The proteins were visualized by staining with HS-CBB gel staining kit (Shanghai Applied Protein Technology Co. Ltd, Shanghai, China). The staining was performed by employing the protocol suggested by the manufacturer.

2-DE and gel staining: IEF was carried out with 17 cm, pH 3-10 IPG strips (non-line) according to the manufacturer's instructions, with minor modifications. The 2-DE gels were run on two separated occasions for each sample. The IPG strips were located in the IPG ceramic holders and rehydrated overnight with 60 μg of the sample, which was solubilized in 320 μL of a solution that contained 8 M urea, 2 % CHAPS, 18.3 mM DTT, 0.5 % v/v IPG buffer (pH 5-8) and a trace of bromophenol blue. The proteins were separated by the IEF system using a programmed voltage gradient at 20 °C. After overnight rehydration, the voltage was set at 500 V for 1 h, increased to 4000 V with a 3 h linear gradient and then was increased further to 10000 V for 5.5 h. The samples were maintained at 8000 V until a total run of 47.5 kVh was accomplished (about 9.5 h in total). After IEF, the strips were equilibrated for 15 min in 10 mL of equilibration buffer (6 M urea, 2 % SDS, 0.05 M *Tris*-HCl (pH 8.8), 30 % glycerol, 1 % DTT) and after that, they were transferred for a further 15 min into equilibration buffer that contained 2.5 iodoacetamide instead of DTT. The equilibrated IPG strips were transferred onto 12.5 % uniform second-dimensional polyacrylamide gels and electrophoresis was carried out in vertical uniform slab SDS-PAGE at 25 mA per gel for about 5.5 h. Protein spots in the gels were visualized by silver staining using conventional protocols, with minor modifications. Briefly, the gels were fixed overnight in 40 % ethanol and 10 % acetic acid in water. After the gels were washed twice with 30 % ethanol in water, they were incubated for exactly 1 min in 0.05 % w/v NaS_2O_3. The gels were then washed twice with water for 20 s each and incubated for 20 min in 0.2 % w/v $AgNO_3$. The gels were washed three times for 20 s each in water and developed with 3 % w/v Na_2CO_3 in 0.05 % v/v formaldehyde in water until the desired contrast was reached (usually after 2.5 min). The reaction was stopped by the addition of 0.5 % acetic acid and incubation for 20 min.

Gel image and data analyses: After the staining, 1D and 2D gels were scanned using Fluor-STM Multimager (Bio-Rad, USA) and the images were analyzed using PDQuest 7.1.1 (Bio-Rad, USA). The data were analyzed using one-way analysis of variance followed by least significant difference test (SPSS11.0 statistics software). The results were expressed as the mean ± standard error of the mean (SEM). A statistically significant difference was considered to be present at $p < 0.05$.

RESULTS AND DISCUSSION

Evaluation of efficient of the different concentration methods: The total protein content was used to investigate the reproducibility and percentage of proteins left after concentration. The percentage of serum proteins in pre- and after-concentration is presented in Table-1. No significant differences were observed between untreated serum and treated samples in total quantity of proteins. From the table we may find that among all of three methods, freeze-drying and ultrafiltration have a high concentration efficient and are able to satisfy the requirements of the next 2-DE experiments. But the percentage of concentrate recovery has shown slightly different result. Freeze-drying and ultrafiltration delivered a higher protein recovery comparing with kit which was no less than 90 %. Moreover, the recovery of the method freeze-drying was especially high and reached to 96.26 % on concentration with depleted serum samples with a low CV value. ReadyPrep 2-D cleanup kit, however, had a low efficient of recovery; it could be caused by a protein loss that happened during the kit treatment process and most likely on account of the washing steps of the protocol.

TABLE-1
COMPARISON OF CONCENTRATE EFFICIENCY OF DIFFERENT METHODS WITH SOLUTE CRUDE SERUM SAMPLES AND HIGH ABUNDANT PROTEIN DEPLETED SERUM SAMPLES*

	Crude serum			High abundant proteins depletion serum		
	Ultrafiltration	Freezing-dry	ReadyPrep 2-D cleanup kit	Ultrafiltration	Freezing-dry	ReadyPrep 2-D cleanup kit
Average solute concentration (μg/μL)	1.26	1.26	1.26	0.37	0.37	0.37
Concentrate volume (μL)	132.25±5.17	150**	150**	146.50±6.20	150**	150**
Concentration after treatment (μg/μL)	8.63±0.07	7.92±0.06	7.37±0.07	2.36±0.06	2.40±0.03	2.15±0.07
Concentrate recovery (%)	90.51±0.03	94.23±0.01	87.79±0.01	93.00±0.02	96.26±0.01	87.26±0.03
CV (%)	7.51	1.40	1.80	5.25	2.39	6.39

*The results were expressed as the mean± standard error of the mean (SEM); **Resuspend concentrated pellets by freezing-dry and ReadyPrep 2-D cleanup kit by adding 150 μL PSB.

1-DE and 2-DE of crude and depleted serum samples: In the serum proteomics studies, the proteins concentration usually is a common and important step, especially in 2-DE or 2D-DIGE experiments. Ultrafiltration, as a common used method, has

been applied in several researches[8,9]. Freeze drying (lyophilization) is a common technology extensively used in the genetic engineering pharmaceutical field[10]. From this display of 1-DE and 2-DE gels (Figs. 1 and 2), Proteo Extract albumin/IgG removal kit has provided an efficient method of IgG and albumin depletion. Similarly, the clear proteins bands were presented by 1-DE and 2-DE gels *via* these three concentration strategies. It revealed that serum proteins after concentrations were available for the research of serum proteomics. Furthermore, the proteins showed clearly in the 2-DE images that serum proteins after treatment by these three methods of concentration had been free of salt and other disturbing factors, such as ionic detergents, nucleic acids, lipids and the like that could interfere with the 2-DE[7].

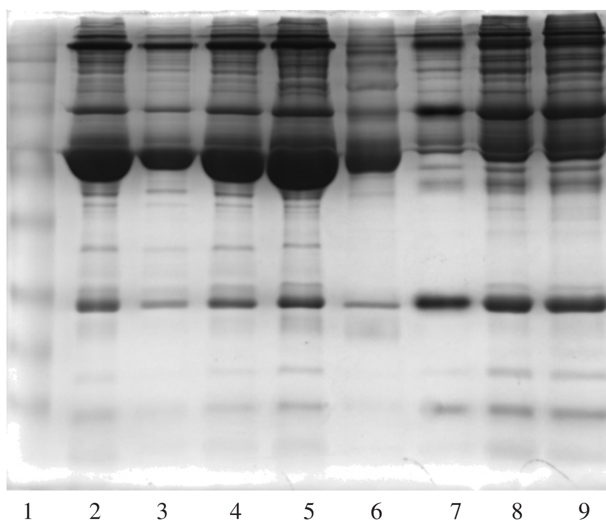

Fig. 1. SDS-PAGE of proteins from human serum and depletion serum by different concentration methods, stained with collodial blue. (1) A series of molecular weight standards as a negative control. (2) Untreated crude serum. (3) Crude serum concentrated by ReadyPrep 2-D cleanup kit. (4) Crude serum by concentrated ultrafiltration. (5) Crude serum by concentrated freeze-drying. (6) Diluted serum ready for high abundance protein depletion as a control. (7) Depleted serum concentrated by ReadyPrep 2-D cleanup kit. (8) Depleted serum concentrated by ultrafiltration. (9) Depleted serum concentrated by freeze-drying.

Image analysis using PDQuest was performed in triplicates of crude serum and depleted serum 2-D gels. Due to the large differences in protein pattern between crude and depleted serum gels, only about 50 % matching was achieved between these two sets of gels. Matching was therefore performed in two separate match sets, one for crude serum 2-D gels and one for depleted serum 2-D gels, using the same spot detection criteria. The detected spots of crude serum on the images of ReadyPrep 2-D cleanup kit, ultrafiltration and freeze-drying concentrate methods were 256 ± 21, 267 ± 17, 271 ± 7, respectively by software analysis and the images of depletion serum were 303 ± 16, 314 ± 8 and 330 ± 13, respectively. Although

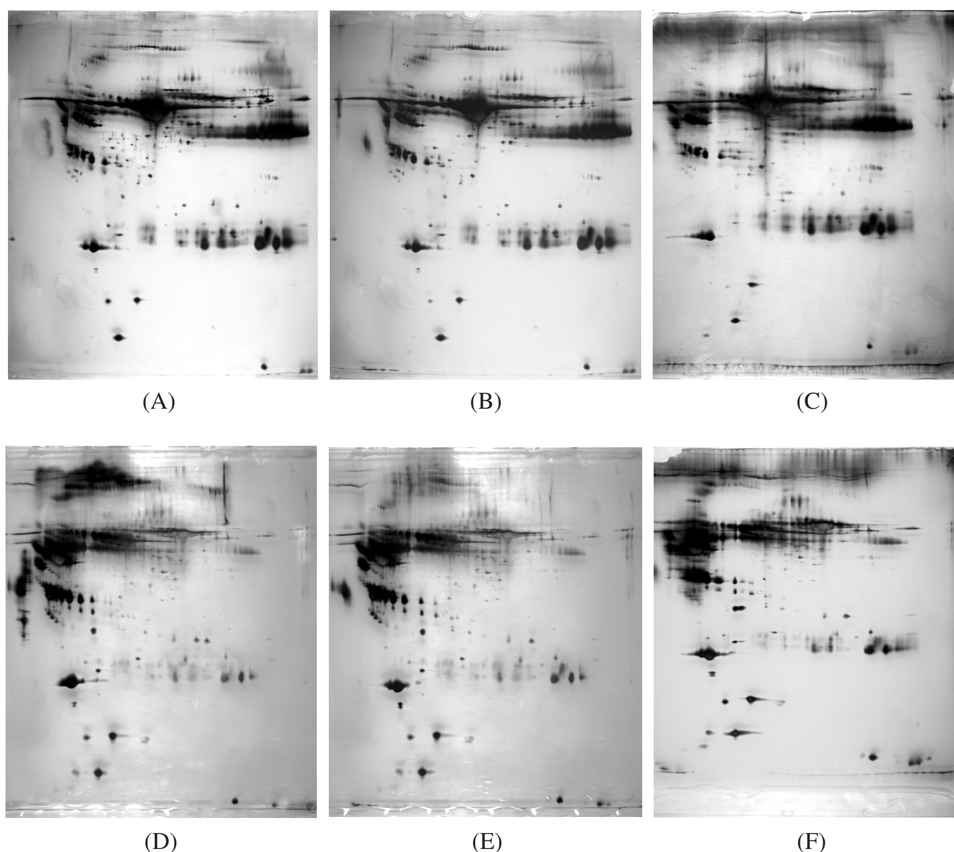

Fig. 2. Two-dimensional electrophoresis analysis of human serum (A, B, C) and of high abundance depletion serum (D,E,F) following different concentration methods with freeze-drying (A and D), ultrafiltration (B and E) and ReadyPrep 2-D cleanup kit (C and F), respectively. A 60 ug of serum was used in each case. Analysis was performed on pH 3-10 NL IPG strips, (17 cm) in the first dimension and 12% SDS gels in the second dimension. The gels were stained with silver staining.

freeze-drying methods showed a large number of displayed spots, statistics hadn't reached a significantly different results ($p > 0.05$). Lots of the same protein spots on the images of the three methods showed a different intensity, which could be caused by the different concentratration strategies. However all images had a good reproduction by the same concentration methods. These suggest that all of these three concentratration methods have a good compatibility with the serum proteomics technique of 2-DE. In addition, uniform concentratration methods during one experiment should be designed and conducted for avoiding bias of experiments or else the results of the experiments would be affected. Therefore, uniformed confrontation method should be applied during one experiment in order to avoid bias and the possible impact to the result.

Evaluation of manipulation of different concentration methods: Table-2 showed some parameters of manipulation for the different concentration methods. Comparing these three concentratration strategies, freeze-drying and ultrafiltration have less manual procedures involved, by which the concentrated proteins of untreated and treated serum samples showed fewer losses. On the contrary, the serum samples handled by ReadyPrep 2-D cleanup kit biases much more on the results of concentration of proteins, which could be attributed to more manual operations. Therefore, uniformed experimental standards are especially important. However no special instruments are required for the kit method, it is inexpensive and the supercentrifuge is one of the basic standardized equipments in most laboratories. Moreover, the cost of ultrafiltration concentration depends on the instruments of ultrafiltration such as centrifugal filter devices. In a contrast, the main equipment of freeze-drying machine is necessary for the freeze-drying methods. Considering the time needed and the treating volume capacity of these three methods, ultrafiltration method performed a high volume that reached about 15 mL by Ultra-15 centrifugal filter device in present research with a shorter time contribute to the concentrate principal that the membranes used in ultra devices are characterized by nominal molecular weigh limit, *i.e.*, their ability to retain molecules above a specified molecular weight. Unfortunately, freeze-dry takes longer time and process less efficiently because freeze-dry is influenced by its small volume of treatment with a small contain caliber connect with ultra low temperature of air in this experiment. The time cost by ReadyPrep 2-D cleanup kit is in the middle comparably with a small mount of volume treatment. In addition ultrafiltration performs very well, however, it is often labour demanding to follow the concentrate volume, the centrifugation steps are usually lengthy, the filters are often blocked. As for the concentrated samples-serum-especially being solute serum samples, perform a well effect by ultrafiltration, which are uneasy be blocked. And proteins after concentration of freeze-drying reserve a good activity, which is the significant advantage. Finally, an important advantage of freeze-drying worth mentioning is that, it has very little impact on protein activities.

TABLE-2
MAJOR CHARACTERISTICS OF THE MANIPULATED EVALUATION OF CONCENTRATE STRATEGIES

Concentrate methods	Complexity degree	Time needed*	Operable quantity	Device and machine needed	Affected factors
ReadyPrep 2-D cleanup kit	Complex	4 h	1-500 μg (optimal 100 μL)**	No	Many
Ultrafiltration by Ultra-15 centrifugal filter device	Simple	1 h	15 mL	Super centrifuge	Few
Freeze-drying by VirTis BT 3.3 EL benchtop freeze dryer	Simple	16 h	Varied with different conditions***	Freeze-dryer	Seldom

*The spending time was estimated by our experiments and devices according to different concentration methods; **For a 1.5 mL microcentrifuge tube, 1-500 μg of protein in a final volume of 100 μL is a recommendation of the protocol; ***The more volume to be treated needs more time consuming by the freeze-drying machine.

Conclusion

Recently, applications of serology and proteomics based techniques in the medical and chemical fields have been reported. A lot of researches are to find some tumor markers in serum by serum proteomics[11,12]. Suitable pretreatments and preparations for serum, therefore, are the keys to a successful research. Indeed, the concentration of some treated serum proteins is a basic pretreatment step preparing for the subsequent experiments[13,14].

Present study conducted a comprehensive investigation of the available concentration strategies which will make contribution to future proteome studies of biological fluids. Three different methods for concentration of crude and high abundant protein depleted serum proteins were evaluated in terms of operation process, the recovery effect and reproducibility. Based on 1-DE and 2-DE analysis, quantification of total protein and albumin concentrations, as well as the images analysis with 2-DE, it is concluded that the freeze-drying and ultrafiltration methods appear to be superior to ReadyPrep 2-D cleanup kit, in terms of efficiency and reproducibility. 2-DE analysis of crude and depleted serum samples demonstrated a good compatibility with 2-DE technique of serum proteomics in a highly reproductive fashion. In conclusion, present results show that these three strategies are the efficient methods to reach into the areas in studies of the serum proteome.

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