Broad-spectrum Four-dimensional Orthogonal Electrophoresis: A Novel Comprehensively Feasible System for Protein Complexomics Investigation*

Xiaodong Wang‡, Fenjie Li‡, Gaoguang Song‡, Shuai Guo‡, Hui Liu‡, Guoqiang Chen‡, and Zhili Li‡§

The major challenge of "protein complexomics" is to separate intact protein complexes or interactional proteins without dissociation or denaturation from complex biological samples and to characterize structural subunits of protein complexes. To address these issues, we developed a novel approach termed "broad-spectrum four-dimensional orthogonal electrophoresis (BS4-DE) system," which is composed of a nondenaturing part I and denaturing part II. Here we developed a mild acid-native-PAGE to constitute part I, together with native-thin-layer-IEF and basic-native-PAGE, widening the range of BS4-DE system application for extremely basic proteins with the range of pI from about 8 to 11 (there are obviously 1000 kinds of proteins in this interval), and also speculated on the mechanism of separating. We first proposed ammonium hydroxide-ultrasonic protein extractive strategy as a seamless connection between part I and part II, and also speculated on the extractive mechanism. More than 4000 protein complexes could be theoretically solved by this system. Using this approach, we focus on blood rich in protein complexes which make it challenging to sera/plasma proteome study. Our results indicated that the BS4-DE system could be applied to blood protein complexomics investigation, providing a comprehensively feasible approach for disease proteomics. Molecular & Cellular Proteomics 11: 10.1074/mcp.M111.012450, 786–799, 2012.

In the postgenomic era, protein complexomics highlights its prominent role in proteomics. Characterization of biologically important protein complexes can provide an integrative view of the protein-protein interactive networks that reveal protein function and biological behavior. Despite enormous progresses in two-step affinity purification (1), comprehensive two-hybrid (2, 3), high-throughput yeast two-hybrid (4, 5), co-immunoprecipitation (co-IP) (6), and high-throughput coafinity purification followed by mass spectrometry (MS) (7) for separating and characterizing protein complexes, the multi-step process is liable to lead to the dissociation and even denaturation of protein complexes. Convenient approaches that could be used for direct study of protein complexes are, as yet, lacking.

Various forms of mild electrophoresis have for some time been the best tools for directly analyzing protein complexes, such as the charge shift techniques: blue native electrophoresis (BNE)1 (8) and high resolution clear native electrophoresis (hrCNE) (9), offering clear advantages for hydrophobic protein complex analyses, especially for membrane protein complexes. However, BNE is not suitable for detergent-labile assemblies’ analyses and in-gel catalytic activity assays because of the use of anionic detergents and Coomassie brilliant blue (CBB) dye. Also, hrCNE has been proven successful in some but not in all aspects (9). Clear native electrophoresis (CNE) (10), in contrast, has only recently been recognized as a valuable and milder technique to isolate and functionally investigate multiprotein complexes (11, 12). However, lower resolution and narrower separation of basic proteins or complexes are major limitations of CNE compared with BNE or hrCNE (13). Nondenaturing micro-two dimensional electrophoresis (micro 2-DE) was developed later to overcome the lower resolution of CNE (14). Although it has been used widely in plasma and cytoplasm protein complex investigations (15–

1 The abbreviations used are: BNE, blue native electrophoresis; BS4-DE, broad-spectrum four-dimensional orthogonal electrophoresis; FTICR MS, Fourier transform ion cyclotron resonance mass spectrometry; co-IP, co-immunoprecipitations; DOC, sodium deoxycholate; hrCNE, high resolution clear native electrophoresis; CBB, commassie brilliant blue; native-tl-IEF, native-thin-layer-IEF; Hp, haptoglobin; α1-AAG, α1-acid glycoprotein; IgG, immunoglobulin G; CP, Proteasome 20S core particle; suc-LLVY-AMC, Succinyl-Leu-Leu-Val-Tyr-7-amino-4-methylcoumarin; PIEFF, post-IEF focusing; PMTs, post-translational modifications; HAP, Hp-associated proteins; TRP, theoretically resolving power.

Competing financial interests: The authors declare no competing financial interests.
17), it is still not suitable for basic protein analysis with a pH above the pH of the gel.

Because of the diversity of protein complexes in vivo, including numerous weak interactions, a means of mild, convenient, and broad-spectrum characterization for protein complexes under native conditions without dissociation is desired. Meanwhile, as practical exploration of complexomics should also incorporate intact complexes and dissociated subunit information, so, we developed a novel approach termed the broad-spectrum four-dimensional orthogonal electrophoresis (BS4-DE) system, based on our previous four-dimensional electrophoresis (4-DE) system (18), which is mainly composed of two parts: 1st-/2nd-DE: native-thin-layer-isoelectric focusing (IEF) (native-tl-IEF)/acidic-basic-native-PAGE (nondenaturing part I), and 3rd-/4th-DE: denaturing-IEF/SDS-PAGE (denaturing part II) (Fig. 1). The optimization of the BS4-DE system can be described as follows. (1) We developed a mild acidic-native-PAGE to constitute 2nd-DE together with basic-native-PAGE, extending the range of the applicable BS4-DE system pH range from \(-3.0\) to 8.4 and from \(-3.0\) to 11.0, and also speculated on the separating mechanism of 2nd-DE\textsubscript{acidic}: acidic-native-PAGE. (2)

**Fig. 1.** Schematic for broad-spectrum four-dimensional orthogonal electrophoresis (BS4-DE) system. Protein complex mixtures are separated by 1st-DE: native-tl-IEF, followed by basic buffer equilibrium. And then 2nd-DE: acidic-basic-native-PAGE separation is carried out. Subsequently, the protein complex spots are dug out and suffered by NH\(_3\)-H\(_2\)O-ultrasonic protein extraction before 3rd-/4th-DE: denaturing-IEF/SDS-PAGE separation.
We first selected ammonium hydroxide (NH₃·H₂O) to extract protein from gels as a connection of part I and part II without gaps, and speculated on the extraction mechanism. Using this approach, we focused on blood rich in protein complexes or interactive proteins, which make it challenging to sera/ plasma proteome study. We displayed differential protein distributions between serum and plasma in part I separation, and provided a simple and convenient method for haptoglobin (Hp) complex phenotype classification. In addition, we characterized differential protein complexes between normal and chronic disease sera by part I separation and MS analysis. The elaborately differential gel-spectra of Hp, α1-acid glycoprotein (α1-AG), and immunoglobulin G (IgG) subunits between normal and disease sera were characterized by part II mapping. Our results indicated that the BS4-DE system was a comprehensively feasible approach for blood protein a complexomics investigation, especially for disease proteomics.

**EXPERIMENTAL PROCEDURES**

**Materials and Instruments—**Ten types of standard protein were purchased from Sigma-Aldrich and used without further purification (supplemental Table S1). Normal sera and plasma were obtained from donor blood in our laboratory. The sera of pancreatic cancer, gastric cancer, esophageal cancer, thyroid cancer, leukemia, diabetic, and hypertensive patients were selected from clinical examination of the laboratory in Peking Union Medical College Hospital and Peking University People’s Hospital (Beijing, China) with patients’ informed consent, and all the cases were confirmed by clinical diagnosis. Amphotiles (pH 3.0–9.5, 3.5–10.0, 5.0–7.0, 6.0–9.0) were purchased from the Academy of Military Medical Sciences. Succinyl-Leu-Leu-Val-Tyr-7-amino-4-methylcoumarin (suc-LLVY-AMC) was obtained from the Academy of Military Medical Sciences. Succinyl-Leu-Leu-Val-Tyr-7-amino-4-methylcoumarin (suc-LLVY-AMC) was obtained from BIOMOL. α-Cyano-4-hydroxyquinamic acid (CHCA) was from Sigma-Aldrich. Pharmalyte IPG buffer 3.0–10.0 was from GE Health Care. Sequencing-grade trypsin was purchased from Roche diagnostics. All other chemicals were obtained from Merck. Etan IPGphor 3 IEF System and Ettan DALTsix System were obtained from GE Healthcare. Fourier transform ion cyclotron resonance MS (FTICR MS) (APEX-Qe 9.4T) was from Bruker Daltonics Inc. (Bremen, Germany).

**The 1st-DE: Native-tl-IEF—**Herein, we employed tl-IEF as the first dimension electrophoresis to be compatible with the IEF demands of 2nd-DE and extended the range of application to meet extremely basic proteins separation. Basic-native-PAGE was employed for the acidic protein or protein complex separation. Briefly, the unstained strips from 1st-DE gel were transferred to equilibration buffer (0.01 M Tris/0.076 M glycine, pH 8.3) for 30 min equilibration, shaking slightly. Then, these gel strips were placed into the slot of two glass plates around 10 mm away from the top of the glass plates. A 4–17% (v/v) linear gradient gel was selected as the separating gel based on previous study (19). When the separating gel solidified (about 50 min), 4% (w/v) stacking gel was overlaid to imbed the focused gel strips. Twenty-five mM Tris/192 mM glycine was used as electrode running buffer, and electrophoresis was run at 15 mA/gel for 45 min, followed by 25 mA/gel for 5 h at 4 °C. A HMW native protein mixture (66–669 kDa) (GE Healthcare) was used as a marker. Acidic-native-PAGE was performed for extremely basic protein or protein complex analysis. Briefly, the parallel gel strips equilibration was performed in a new optimized buffer for 20 min, containing 0.01 M Tris, 0.076 M glycine, and 0.03 M β-alanine at pH 9.0. Acetate-KOH (pH, 4.8) and acetate-KOH (pH, 6.8) buffers were applied to replace Tris borate-EDTA (TBE) buffer (pH, 8.4) in the separating gel and Tris-HCl buffer (pH, 7.5) in the stacking gel for acidic gel preparation, respectively. Furthermore, acetate-β-alanine (pH, 4.8) was used as running buffer instead of Tris-glycine (pH, 8.4). It is noteworthy that the polarity should be reversed to avoid the loss of basic sample during the electrophoresis.

**Molecular & Cellular Proteomics 11.9**
the bromphenol blue migrated to the bottom of the gels. PageRuler Unstained protein Ladder (SM0661) (Fermentas) was used as marker.

The 3rd-/4th-DE: Denaturing-IEF/SDS-PAGE—Prior to denaturing-IEF, about 45 μL of protein extractive solution was mixed with 450 μL of the rehydration solution, and then subjected to IPG buffer (pH 3.0–10.0, nonlinear, GE Healthcare) and Destreak (GE Healthcare) treatments at the final concentrations of 1.2 and 0.5% (v/v), respectively. IPG DryStrips (pH 3.0–10.0, nonlinear, 24 cm, GE Healthcare) were applied to Ettan IPGPhor 3 IEF System. After rehydration for 20 h at 40 V at 20 °C, IEF was conducted for 2 h each at 100, 200, 500, 1000 V, and 2 h at 1000–8000 V in gradient mode, followed by 8000 V for a total of 144 kVh. After IEF separation, the gel strips were equilibrated for 15 min in the buffer containing 50 mM Tris-HCl, pH 6.8, 7 M urea, 30% (v/v) glycerol, 2% (w/v) SDS, 1% (w/v) DTT, and a trace of bromphenol blue, followed by 15 min in the same buffer except that 2.5% (w/v) IAA was used instead of 1% (w/v) DTT. The 4th-DE: SDS-PAGE was carried out on Ettan DALTsix System at 12% (w/v) separating gel (205 mm × 255 mm × 1.0 mm) at 40 mA. Proteins were visualized by CBB. Gels were scanned and stored at −20 °C until use.

The Acidic-Native-PAGE Gel Property Evaluated by Proteasome 20S Core Particle (CP) Hydrolysis Activity Assay—To confirm that the proteins or protein complexes separated by the 1st-/2nd-DE remain biologically active, the CP purified from the SW1990 cell line cytoproteins or protein complexes separated by the 1st-/2nd-DE remained largely unaltered activity. The CP purified from the SW1990 cell line cytoproteins or protein complexes separated by the 1st-/2nd-DE remained largely unaltered activity. The CP purified from the SW1990 cell line cytoproteins or protein complexes separated by the 1st-/2nd-DE remained largely unaltered activity.

RESULTS

Acidic-Native-PAGE, an Attractive and Essential Component of BS4-DE System—We employed 10 types of standard proteins with different MWs and pI values (supplemental Table S1) to evaluate the performance of 1st-/2nd-DEbasic in a previous study (18) in a previous study (18) in a previous study (18). Despite the higher resolution of 1st-/2nd-DEbasic separation compared with 1st-/2nd-DEbasic, basic-native-PAGE, 1st-/2nd-DEbasic is still incompatible with extremely basic proteins (pI above the pH of the gels), such as cytochrome c (pI, 10.0–10.5) and lysozyme (pI, 11.0) (Fig. 2A). Actually, these proteins catch positive charges during electrophoresis, and move to the anode. To address this bottleneck we developed a mild acidic-native-PAGE combined with native-IEF and basic-native-PAGE to constitute part I, termed as native-IEF/acidic-basic-native-PAGE (1st-/2nd-DE). For acidic-native-PAGE preparation, acetate-KOH (pH, 4.8), acetate-KOH (pH, 6.8), and acetate-β-alanine (pH, 4.8) buffers were employed to replace Tris borate-EDTA (TBE) buffer (pH, 8.4) in separating gel. Tris-HCl buffer (pH, 7.5) in stacking gel, and Tris-glycine in running buffer (pH, 8.4), respectively. Notably, the polarity should be reversed during the electrophoresis process to avoid the loss of basic sample. As shown in Fig. 2B, cytochrome c and lysozyme were successfully separated by native-IEF/acidic-native-PAGE (1st-/2nd-DEacidic). We employed normal serum to evaluate the 1st-/2nd-DE (Figs. 2C, 2D). These results indicated that acidic proteins or complexes presented better resolution in 1st-/2nd-DEacidic meanwhile, basic proteins or complexes were suitable for 1st-/2nd-DEacidic separation. For instance, a series of IgG spots could be found on 1st-/2nd-DEacidic gel, which were absent on 1st-/2nd-DEbasic gel. All the spots were unambiguously identified by matrix-assisted laser desorption/ionization-FTICR MS (MALDI-FTICR MS) (supplemental Table S2 and Supplement Data-Annotated Spectra). Remarkably, this novel mild acidic-native-PAGE widened the BS4-DE system’s applicable pI range from ~3.0 to 8.4 and from ~3.0 to ~11.0.

NH₃₂H₂O-Ultrasonic Protein Extractive Strategy as a Seamless Connection Between Part I and Part II—We first proposed and characterized NH₃₂H₂O-ultrasonic protein extractive strategy as a seamless connection between part I and part II (supplemental Figs. S1, S2) instead of sodium hydroxide (NaOH) extraction to eliminate thoroughly the application barrier caused by salt (18, 22).

We optimized this strategy using re-electrophoresis of BSA after extraction (supplemental Fig. S1). We chose 0.166% NH₃₂H₂O, 0.01 m NaOH, 0.166% NH₃₂H₂O, and 0.01 m NaCl for BSA in-gel extraction, respectively, followed by re-electrophoresis to detect the recoveries (supplemental Fig. S1a, left). We found that NH₃₂H₂O can be used for protein extraction similar to NaOH. Conversely, NH₃₂H₂O and NaCl showed the negative results. Therefore, it could be easily proven that OH⁻
was the reactive ion for extraction (supplemental Fig. S1a, right). It is worthwhile to mention that the 53.3% recovery of 0.166% NH$_3$/H$_2$O extraction is closely similar to that of 0.01 M NaOH extractive recovery (52.0%) reported by a previous study (22), suggesting that NH$_3$/H$_2$O-ultrasonic extraction was a feasible and efficient strategy. We have evaluated the extractive recoveries of BSA using different concentrations of NH$_3$/H$_2$O to optimize the NH$_3$/H$_2$O extractive concentration (supplemental Fig. S1b). The results indicated that the recovery was related to the concentrations of NH$_3$/H$_2$O. The optimized concentration of NH$_3$/H$_2$O was assigned as 0.25% with the BSA recovery of 95.5%. We also evaluated the efficiency of 0.25% NH$_3$/H$_2$O extraction of other proteins with different MWs and pI's, such as cytochrome c, lysozyme, transferrin, thyroglobulin, and fibrinogen (supplemental Fig. S1c and supplemental Table S1). These results implied that the 0.25% NH$_3$/H$_2$O could meet with broad-spectrum protein extraction, except for larger molecular weight protein (MW $>$ 669.0 kDa), e.g. thyroglobulin. It also suggested that the properties of proteins, such as structure, post-translational modifications, and hydrophilicity/-phobicity should be taken into full consideration to increase the recovery efficiency. The comparisons of the recoveries of lane 1, lane 4, lane 5, lane 15, lane 16, and lane 28 showed the intra-/intergel reproducibility of NH$_3$/H$_2$O-ultrasonic protein extractive strategy (supplemental Fig. S1).

**Fig. 2. Establishment and evaluation of 2nd-DE: acidic-basic-native-PAGE.** A, Ten kinds of standard proteins were employed to evaluate the performance of 1st-/2nd-DE$_{basic}$: native-til-IEF/basic-native-PAGE (Supplemental Table S1), all the proteins could be separated by 1st-/2nd-DE$_{basic}$ except lysozyme and cytochrome c, indicating that basic-native-PAGE was an incompatible approach for extremely basic proteins with pI above the pH of gel. B, Acetate-KOH and acetate-β-alanine were applied to establish acidic-native-PAGE for extremely basic proteins separation. Cytochrome c and lysozyme were successfully separated by this acidic-gel. Hemoglobin was used as a reference. C, Evaluation of 1st-/2nd-DE was performed by 8 μL normal serum separating. 20 and 6 bands were separated by 1st-DE$_{basic}$ and 1st-DE$_{acidic}$ respectively. In contrast, 45 spots were shown on 1st-/2nd-DE$_{basic}$ gel and 18 spots were observed on 1st-/2nd-DE$_{acidic}$ gel, which indicated that acidic proteins or complexes displayed better resolution in 1st-/2nd-DE$_{basic}$. Meanwhile, basic proteins or complexes were suitable for 1st-/2nd-DE$_{acidic}$ separation. All the spots were identified by MALDI-FTICR MS (Supplemental Table 2).
Although NaOH protein extraction has been proven to be an efficient approach as a connection for 4-DE (18), the high concentration salt will destroy the IEF process of 3rd-DE. In this study, the NH$_3$H$_2$O protein extractive solution could be really subjected to the 3rd-DE separation directly after being lyophilized, and the restriction on volume of the extractive solution could be thoroughly dispelled because of the volatility and alkalinity of NH$_3$H$_2$O. The volatility of NH$_3$H$_2$O at different concentrations was also evaluated by pH test strips (supplemental Fig. S2a). We observed that all kinds of NH$_3$H$_2$O solutions gradually changed to neutral pH during the lyophilization, and the alkaline-neutral inflection points were marked as red line (supplemental Fig. S2a). It is noteworthy that previous MS analyses indicated that the alkaline cleavage of the peptide bond was rather restricted (22, 23) and the first-order rate constants of the β-elimination reactions of protein covalent bonds caused by alkaline at the most susceptible site were less than 0.1/h in 0.1 M NaOH solution, indicating that the protein degradation can be negligible in 0.25% NH$_3$H$_2$O, which is consistent with our study. Briefly, we employed BSA to be dealt with different concentrations of NH$_3$H$_2$O (0.25%, 2.5, and 5%) at different treatment times (1 h, 12 h, and 24 h), followed by electrospray ionization-FTICR MS (ESI-FTICR MS) analysis for precise mass detection, and no obvious discrepancy was found (supplemental Fig. S2b), indicating that NH$_3$H$_2$O was a suitable reagent for protein in-gel extraction.

**Blood Protein Complexomics Profiling by BS4-DE System**—To evaluate the general utility of BS4-DE, we focus on blood rich in protein complexes or interactional proteins, which make it challenging for a sera/plasma proteome study. These results showed an unambiguous variance of fibrinogen between serum and plasma on part I maps (supplemental Fig. S3a), providing a potential application for clinical examination. The phenotypic classification of Hp complexes could also be directly obtained by 1st-/2nd-DE$_{basic}$ separation in a new perspective without the need of affinity purification before electrophoresis. Therefore, this simplified analysis procedure was favorable in maintaining the intact Hp complex. Hp consists of two different polypeptide chains, the α-chain and the β-chain (supplemental Fig. S3b), forming three phenotypes of Hp: Hp 1–1, Hp 2–1, and Hp 2–2 based on a previous study (24). In this study, four phenotypes of Hp were found in 1st-/2nd-DE$_{basic}$ separation (supplemental Fig. S3c), including Hp 2–2, Hp 2–1, Hp 1–1, and Hp 0. All of these phenotypes could be isolated from normal sera except Hp 0, which was found in Hp-deficient leukemia serum (supplemental Fig. S3c), consistent with a previous study (25). The homozygote Hp 1–1 is composed of α1-chain and β-chain with formula (α1β)_1 and Hp 2–2 is composed of α2-chain and α-chain with formula (α2β)_2(n = 3, 4, 5, ...). The heterozygote Hp 2–1 consisted of α1-chain, α2-chain, and β-chain with formula (α1β)α2β$_{n=2}$ (n = 3, 4, 5, ...). Both Hp 2–2 and Hp 2–1 were characterized in polymerization, comprising higher molecular mass forms (trimer, tetramer, pentamer ...) in agreement with a previous study (26). The components of Hp 2–2 and Hp 2–1 could be easily characterized by part II separation. We employed seven kinds of chronic disease sera, including pancreatic cancer, gastric cancer, esophageal cancer, thyroid cancer, leukemia, diabetes, and hypertension to explore the general applicability of BS4-DE system in disease proteomics (Figs. 3, 4). Normal sera, as a reference, and each disease sera was pooled from eight clinically diagnosed cases, containing 4 Hp 2–1 and 4 Hp 2–2 phenotypes, to reduce individual differences and avoid the systematic residuals caused by individual experiment. Six variable regions (VR 1–6) and IgG distribution are observed on part I maps, suggesting that there are differential proteome between normal and disease sera (Fig. 3). The spots of Hp N2, Hp G2 and Hp L2, and IgG NVIII, IgG GVIII, and IgG LVIII were selected for SDS-PAGE separations, followed by Hp β subunit and IgG heavy chain (γ chain) glycosylation studies (supplemental Fig. S4). Furthermore, the subunits of Hp (N2, G2, G3’, and D2 spots), α1-AG (N9, G9, and D9 spots), and IgG (NIll, GIII, and DVIII spots) were characterized by part II proteomics analysis, respectively (Fig. 4), indicating that BS4-DE was a convenient, valuable strategy for blood proteome research, especially for disease differential protein complexomics analysis. All the spots were unambiguously identified by MALDI-FTICR MS (supplemental Table S3 and supplement Data-Annotated Spectra).

Interestingly, the expressions of all Hp phenotype complexes were up-regulated in cancer and leukemia sera. However, the expressions of Hp 2–1 (e.g. D3, H3) were shown without any obvious change in diabetes and hypertension sera except the H1 spot in hypertension compared with the up-regulated expression of Hp 2–2 (e.g. D3’, H3’) (Fig. 3A). These results indicated that although the hemoglobin (Hb) binding capacity of Hp 2–2 was lowest in Hp phenotypes (25), Hp 2–2 may be a susceptible phenotype for response to lesion or inflammation, especially in diabetes and hypertension patients. Actually, more evidence has become available to demonstrate the deleterious effect of the Hp 2–2 phenotype in diabetes (27) and hypertension (28). Consequently, we could propose that the different expressions of different Hp phenotypes may be the different physiological effects regarding their protective activity against Hb toxicity, which may be valuable for profiling of cancer and noncancerous disease, but these need further study to confirm. We also found obvious differential glycosylation at site 241 asparagine of the Hp β chain tryptic glycopeptide VVLHP$^{241}$NYSQVDIGLIK (supplemental Fig. S4a). In normal serum, the most prominent peak at m/z 3,709.591 contained one N-acetylgalactosaminic acid (sialic acid, Neu5Ac) modification, named as Hp G2F0S1 (G, Gal; F, Fuc; S, Neu5Ac), followed by fucosylated peak at m/z 3855.570 (Hp G2F1S1), and the third intensity peak at m/z 3418.568 without Neu5Ac and fucose modification (Hp G2F0S0). All the glycopeptide peaks were confirmed according to a previous study (29).
relative intensity distribution of peaks changed in gastric cancer and leukemia. These results indicated that part I of BS4-DE was a perfectly mild approach for protein or protein complex separation and PTM analysis. Furthermore, the heterogeneity of HP complex subunits was characterized clearly by part II proteomics analysis coupled with SDS-PAGE separation (Fig. 4A). SDS-PAGE analysis showed that Hp was composed of two chains, α and β. The heterogeneities of α and β chains were shown to be more diverse in disease by 3rd-/4th-DE separation. Fifteen spots, 21 spots, 21 spots, and 24 spots were observed on 3rd-/4th-DE gel maps of N2, G2, G3, and D2, respectively. N2, G2, and D2 spots belonged to Hp 2–1 phenotype, composed of α1, α2, and β chains, and G3’ spot was Hp 2–2 phenotype because of the absence of α1 chain. It is worthwhile to note that two novel Hp-associated proteins (HAP), transthyretin and serum amyloid A protein, were found in N2, G2, and D2 spots, which were absent in G3’ spot, indicating that different Hp phenotypes may reflect different affinity with other proteins and also provide direct proof of the mildness of separation of part I. Importantly, these associations need to be confirmed in future studies combined with other approaches. Notably, the β chains of Hb were found in G2, G3, and D2 spots, proving the unquestionable interaction between Hp and Hb (30), and the absence of Hb in the N2 spot, may be because of the intrinsic low level of Hb in normal serum. The fact that the observed
isoforms of a given subunit display shifted pI values suggested that their presence is more likely the result of PTMs rather than proteolytic cleavages. In future studies, we will explore the Hp detailed modifications and structures to reveal the correlation between Hp phenotypes and disease using enough cases based on BS4-DE system.

The expressions of σ1-AG spots were up-regulated in disease sera (Figs. 3A, 4B). The heterogeneity of σ1-AG was also displayed on part II maps. Two spots of σ1-AG were found in normal serum (N9 spot), and six isoforms of σ1-AG were successfully separated by part II in gastric cancer and diabetes (G9 and D9 spots) (Fig. 4B), respectively, suggesting that diversity of σ1-AG may be related to physiological state.

As shown in Fig. 3B, a series of train-spots of normal serum IgG complexes were obviously observed by 1st-/2nd-DEacidic separation compared with chronic disease IgG blurry spots. We speculated that more IgG complex isoforms were synthesized and assembled in vivo to meet numerous immunoreactions in disease, leading to IgG blurry distribution on 1st-/2nd-DEacidic maps. Previous study has shown that IgG consisted of four subclasses (IgG1, IgG2, IgG3, and IgG4), which all carried N-glycan at N297 site in the conserved CH2 region of their heavy chain (31). As shown in supplemental Fig. S4b, we found a series of glycopeptide peaks in agreement with previous studies (31, 32). The remarkable differences were that the intensity of glyco-
peptides of IgG2 was apparently higher than that of IgG1, indicating that the spots of NVIII, GVIII, and LVIII were mainly composed of IgG2. Actually, previous study suggested that $p_l$ of IgG2 is 7.0–7.5 (33), which was in accordance with VIII spot position ($p_l$ ~ 7.3). The glycopeptide peak at $m/z$ 2805.1, termed as IgG2 GOF1N (N, bisecting GlcNAc), in the GVIII spot spectrum, and peaks at $m/z$ 2796.1 (IgG1 G1F1) and $m/z$ 2805.2 (IgG2 GOF1N) in the LVIII spot spectrum, were higher than their corresponding peaks in NVIII spot spectrum, suggesting that part I may be a convenient approach for simplifying protein PTM study, attributed to the high-performance separation. In addition, the similar spectra among three spots were the indirect proof of stability and reproducibility of 1st-/2nd-DE$_{acidic}$ separation. As shown in Fig. 4C (SDS-PAGE detection), IgG was composed of $\gamma$ chain and light chain ($\kappa$ chain or $\lambda$ chain). The heterogeneity of IgG was characterized by 4-DE (Fig. 4C). As shown in NIII spot, there were 8 $\gamma$ isoforms and 4 $\kappa$ isoforms, similar with GIII spot (9 $\gamma$ isoforms and 6 $\kappa$ isoforms) and DVIII spot (5 $\gamma$ isoforms and 4 $\kappa$ isoforms). The spots with $p_l$ > 7.5 were the subunits of IgG1, and the other spots belonged to IgG2 subunit isoforms. All of the IgG2 subunit isoforms exhibited clearly different $p_l$ values without significant MW variations, suggesting that the difference was likely because of PTMs, rather than the consequence of proteolytic cleavage and/or mRNA splicing events. Remarkably, NIII and GIII spots were rich in IgG1 subunits and rare in DVIII, implying that the DVIII spot may be mainly composed of IgG2 complex isoforms. Moreover, the distribution of DVIII IgG2 subunits was shown at a more acidic region compared with NIII and GIII spots, suggesting that BS4-DE system could be used for IgG1 and IgG2 sub-class preliminary separation and subunit isoform analysis.

**DISCUSSION**

**Optimization of 2nd-DE$_{acidic}$: Acidic-Native-PAGE Conditions**—Proteasome 20S CP was treated with different pH stock solutions and running buffers of acidic-native-PAGE, respectively, followed by hydrolysis activity assay with a substrate of suc-LLVY-AMC to optimize the conditions of 2nd-DE$_{acidic}$ (Fig. 5A). The assay procedure was similar to that described in a previous study (20). We found that the activity of CP could be retained in all kinds of stock solution and running buffer except the separating stock solution at pH 4.3, providing an indirect proof for the mildness of this acidic gel. It is worthwhile to mention that CP hydrolysis activity assay could not be directly performed in acidic-native-PAGE gel, because CP could not gain enough positive charge to form a strong enough electrostatic force for driving the bulky complex into the gel during acidic-native-PAGE process, owing to the intrinsic charge characteristic of CP. Alternatively, an indirect demonstration may be the preferred choice. Taking into the balance between resolution and mildness, the preferential conditions of 2nd-DE$_{acidic}$ were assigned as stacking stock solution (pH, 6.8), separating stock solution (pH, 4.8), and running buffer (pH, 4.8).

**2nd-DE$_{acidic}$: Acidic-Native-PAGE Separating Mechanism**—Interestingly, the same parallel IEF strips treated with different pH equilibrium solutions, presented different protein train-spot patterns on 1st-/2nd-DE$_{acidic}$ maps (Fig. 5B), which indicated that the resolution was positively related to the pH of equilibrium solution. The basic buffer with pH 9.0 seemed to be an optimum equilibrium solution. We speculated on the separating mechanism of acidic-native-PAGE as follows (Fig. 5C). Initially, basic protein occupied a related broad region in gel at the end of native-tl-IEF, and the potential of whole gel could be regarded as zero, showing a potential equilibrium state (E$_0$) (Figs. 5C–5I). E$_0$ would be destroyed by maldistribution of negative charge in gel when the strips were equilibrated with basic buffer (pH 9.0). The whole gel was easily packed with OH$^-$ ions except protein regions because of the protein hydrophobicity, which could reduce the diffusion velocity of OH$^-$ ions (Fig. 5C–II). Unsurprisingly, this transient charge maldistribution would ultimately fade away, which was attributed to the continuous diffusion of OH$^-$ ions to form a homogeneous negative potential in the whole gel and to establish a new potential equilibrium state (E$_1$). The first-post-IEF focusing (1st-PIEFF) of protein grew out of this extrusion force between E$_0$ and E$_1$ transformation (Fig. 5C–III). Similarly, E$_1$ was destroyed by H$^+$ ions from acidic running buffer during acidic-native-PAGE process. H$^+$ ions swiftly occupied the whole gel except protein regions, because of protein hydrophobicity and residual basic equilibrium solutions cushioning (Fig. 5C–IV). Finally, the continuous diffusion of H$^+$ ions would fill up the whole protein regions to form another positive potential equilibrium state (E$_2$) and display the 2nd-PIEFF of protein (Fig. 5C–V, VI). The 1st-/2nd-PIEFF processes showed the unique characteristics of high resolution in 1st-/2nd-DE$_{acidic}$ separation. More importantly, this separating mechanism of acidic-native-PAGE was proven by parallel gels staining (Fig. 5D). First-PIEFF was found on CBB stained IEF gel after basic buffer (pH, 9.0) equilibrium for 30 min, and 2nd-PIEFF was shown on 2nd-DE$_{acidic}$ gels that were stained by CBB at 30 min and 90 min time points during the acidic-native-PAGE process.

**Mechanism of NH$_3$H$_2$O-Ultrasonic Protein Extractive Strategy**—We speculated on the mechanism of protein or protein complex extraction in NH$_3$H$_2$O solution as schemed in Fig. 6. We have provided that basic-native-PAGE and native-tl-IEF/basic-native-PAGE could retain protein physiological activity (18, 34), indicating that these electrophoresis techniques can maintain an intact structure of proteins or complexes (Supplemental Fig. S5a). When a gel was treated with phosphoric acid (H$_3$PO$_4$)-methanol-CBB fast staining solution (pH, 1.5–2.0) for fixing, the hydrophobic regions and positively charged residues of Lys (p$_K^{\alpha}$, 10.5) and Arg (p$_K^{\alpha}$, 12.5) of protein were bound with hydrophobic moieties and/or sulfonate residues (R-$SO_3^-$) of CBB (CBB$^-$), respectively, whereas the negative charges of Glu (p$_K^{\alpha}$, 4.3) and Asp (p$_K^{\alpha}$, 3.9) residues were completely lost. This extremely acidic-methanol environment
Fig. 5. Optimization of acidic-native-PAGE conditions and illumination of acidic-native-PAGE separating mechanism. A, CP hydrolysis activity assay was carried out for the optimization of acidic-native-gel preparation. Each 3 μL CP was dealt with 17 μL stacking stock solution (pH 6.8), 17 μL separating stock solution (pH 4.3, 4.8, 5.3, 5.8, 6.3, and 6.7) and 17 μL running buffer (pH 4.3, 4.8, 5.3, 5.8, and 6.7) for 5 h, respectively, followed by basic-native-PAGE separation and CP activity was assayed using fluorogenic substrates succ-LLVY-AMC in the presence of 0.02% (w/v) SDS. B, Equilibrium solutions with different pH (4.3, 6.3, 7.5, 8.2, 9.0 and 9.5) were employed to soak out Ampholines from IEF gel strips for 30 min before 2nd-DE acidic separation. C, The separating mechanism of acidic-native-PAGE was revealed and illuminated by potential transformation theory. Three equilibrium potential states (E₀, E₁ and E₂) and thrice protein focusing, including native-tl-IEF, 1st-PIEFF and 2nd-PIEFF, were formed in 1st-/2nd-DE acidic process. D, The acidic-native-PAGE separating mechanism could be proven by the parallel 1st-DE and 2nd-DE acidic gels staining. The 1st-PIEFF could be found on native-tl-IEF strip which was stained by CBB after basic buffer (pH 9.0) equilibrium for 30 min, and the 2nd-PIEFF was clearly shown on CBB stained acidic-native-PAGE gels at different time of 2nd-DE acidic process, e.g. 30 min and 90 min.
led to the denaturation of protein, exposing more hydrophobic regions and positively charged residues for CBB binding. The hydrophobic protein-CBB complexes were fixed in gel regions and positively charged residues for CBB binding. The residues of Lys and Arg release the CBB– ions and positive charges, meanwhile, Glu and Asp would get full negative charges during the 0.25% NH₃·H₂O extraction. The right shifting of NH₃·H₂O ionization equilibrium and ultrasonic extraction increase the efficiency protein extraction (Inset: NH₃·H₂O-ultrasonic protein extraction was performed in tube). C, Schematic representation of NH₃·H₂O protein extractive strategy. The right shifting of NH₃·H₂O ionization equilibrium causes the 0.25% NH₃·H₂O pK₄ > 12.5 nearby the residues of Lys, Arg, Glu and Asp, hence CBB− ion could be replaced by OH− completely and all the positively charged amino acids turn to neutral state.

BS4-DE System, a Comprehensively Feasible Approach for Protein Complexomics Investigation—Typically, most current approaches for purification and identification of protein complexes adopt affinity purifications combined with mass spectrometry, such as co-immunoprecipitation (35) and tandem affinity purification (36). However, the time-consuming procedures, nonspecificity binding, and high cost of antibody limit their applications. Alternatively, as a convenient and inexpensive approach, mild electrophoresis may be the preferred choice. The charge shift techniques of BNE and hrCNE have been recognized as efficient microscale techniques to separate and characterize membrane proteins and complexes (8, 9), attributing to the employment of anionic detergents, such as CBB and sodium deoxycholate (DOC). Besides that, CNE is another milder approach for protein complex study compared with BNE or hrCNE. The lack of anionic detergents offers obvious advantages for in-gel catalytic assays, detection of fluorescent labels and retention-ability of physiological protein assemblies. Nevertheless, the limitations of protein aggregation, lower resolution, and narrower separation of basic proteins are also caused by the lack of anionic detergents making CNE lack the advantages of BNE or hrCNE (10). Herein, we propose a novel approach to retain both advantages of CNE and BNE or hrCNE, termed as the BS4-DE system, including a nondenaturing part (part I) and a denaturing part (part II) (Fig. 1). Part I is 1st-/2nd-DE: native-tl-IEF/native-PAGE. A mild acidic-native-PAGE was developed to constitute 2nd-DE together with basic-native-PAGE, widening the applicable pH range of the BS4-DE system from ~3.0 to 8.4 and from ~3.0 to ~11.0 compared with 4-DE (18) (Figs. 1, 2). Higher resolution, milder separation, and broader application could be provided by part I, meanwhile, protein aggregation could be greatly reduced because of the different mechanisms of IEF and native-PAGE separation in part I (Fig. 2). Intact protein complexes or interactive proteins were separated by Part I (Fig. 3 and supplemental Fig. S3), which could not be obtained by traditional 2nd-DE, followed by SDS-PAGE separation and MS analysis to screen the protein complex candidates and inspect post-translational modifications (PTMs) of protein complexes of interest (supplemental Fig. S4). Part II is 3rd-/4th-DE: denaturing-IEF/SDS-PAGE, for profiling subunit distribution of protein complexes or interactive proteins (Fig. 4). In fact, the BS4-DE system is a

Fig. 6. Speculation on mechanisms of NH₃·H₂O-ultrasonic protein in-gel extractive strategy. (A, B) Proteins or complexes are extracted by NH₃·H₂O extractive strategy. The residues of Lys and Arg release the CBB− ions and positive charges thoroughly, and Glu and Asp get full negative charges during the 0.25% NH₃·H₂O extraction. The right shifting of NH₃·H₂O ionization equilibrium and ultrasonic extraction increase the efficiency protein extraction (Inset: NH₃·H₂O-ultrasonic protein extraction was performed in tube).
technical integration deriving from 2nd-DE. No doubt that 2nd-DE is a sufficient method for screening new or differential proteins, but the denaturing conditions would destroy the integrity of protein complexes. Although the BS4-DE system needs a complex process of sample handling, it shows almost all of the advantages of 2nd-DE. More importantly, protein-protein interaction information of protein complexes could be obtained by BS4-DE analysis, indicating that BS4-DE may be an effective approach for 2nd-DE data re-reading and re-exploiting as well as revealing protein interaction networks in the future (supplemental Table S4). As shown in supplemental Fig. S6, we find the resolution capability is BNE<1-DE: native-PAGE<1st-/2nd-DE_{basic} for serum separation, indicating that BNE may be suitable for simple and HMW sample separation, especially for membrane protein complex analysis. The complex and LMW sample would be prone to form serious overlaps during BNE separation (see Overlay regions 1, 2 of BNE gel in supplemental Fig. S6a), because of the excessive negative charge of the protein beyond the gel pore size limitation. Therefore, BS4-DE system may be a preferred choice for complex water-soluble sample separation. The theoretical resolving power (TRP) of BS4-DE was deduced in supplemental Fig. S7, indicating that more than 4000 protein complexes could be theoretically resolved by the 1st-/2nd-DE of BS4-DE. The previous study has shown that the NaOH extractive strategy was an effective connection between part I and part II in 4-DE (18), but the volume limitation blocked the application in low-abundance proteins or complexes in part II analysis because of the high concentration of salt. Consequently, we employed NH₃·H₂O to thoroughly break the constraints of volume because of its attractive properties of alkalinity and volatility and to achieve the seamless connection between part I and part II (supplemental Figs. S1, S2). In comparison with other approaches (supplemental Table S4), such as gel filtration (37) and isopycnic centrifugation coupled with localization of organelle proteins by isotope tagging (LOPIT) (38), BS4-DE not only can be easily used in laboratories with gel equipment, but also can provide relatively high resolution for trace mixture separation in samples containing protein complexes. As a widely used system for the analysis of a protein’s size, gel filtration requires an automated high-pressure pump system (e.g. fast-protein or high-pressure liquid chromatography) for good resolution, and even then the resolution is just sufficient to allow the clear separation of molecules with at least twofold volume. Isopycnic centrifugation-LOPIT has proven useful for the localization of organelle proteins. However, high amounts of protein and high cost are required in isopycnic centrifugation-LOPIT analysis compared with BS4-DE. In addition, the most significant advantage of BS4-DE system is that this method can achieve the separation of protein complexes and characterize protein complex subunits in progressive stages (Figs. 3 and 4), which cannot be obtained completely by single gel filtration or isopycnic centrifugation analysis without coupling with other approaches.

Often, the MS spectra are derived from peptides containing different types of modification, such as oxidation, alkylation, salification, methylation, ethylation, and Schiff base reaction from sample handling (39). In this study, we employed Hp (α), Hp (β), α1-AG, and IgG κ chain for in vitro or in vivo modification detection. After BS4-DE and traditional 2nd-DE separation as well as CBB staining, similar positional protein spots were dug out followed by tryptic digestion and MALDI-FTICR MS identification (supplemental Fig. S8a). Interestingly, the similar MS spectra of 4th-DE and 2-DE displayed a better sequence coverage of 4th-DE than 2-DE, indicating that BS4-DE separation was a more effective approach for peptide identification compared with 2-DE. Therefore, although the various steps of BS4-DE may cause protein in vitro modifications, the resistance of these multisteps to protease treatment could be ignored in this study. It also proved that NH₃·H₂O-ultrasonic strategy was a perfect way for protein extraction, which would not destroy the primary structure of protein and cause additional modifications like nitration. In vitro modifications of protein would cause the change of mass so that they are resistant to identification by MS (39). Consequently, three kinds of modifications (oxidation, +15.99 Da; salification, +21.98 Da, and methylation, +14.03 Da) found in this study (supplemental Fig. S8b), should be taken into consideration as variable modifications in a database search to avoid the modified peptides miss-matching. Previously, we have proven that, as an extensively used solvent, the use of methanol was shown as a “seed incubator” for in vitro methylation during staining (40). In order to reduce or avoid in vitro methylation, we could reduce the staining time and the amount of methanol or choose other methanol-free gel staining methods, such as imidazole-zinc (41) and copper staining (42).

CONCLUSIONS

In this study, we successfully developed a BS4-DE system for preliminarily separation and characterization of blood proteins or complexes. Protein complexes were integrally separated by part I under mild condition, followed by NH₃·H₂O-ultrasonic extraction, and then treated to SDS detection and PTM analysis combined with MS. Moreover, the heterogeneity of interesting complex subunits was characterized by denaturing part II separation. The results showed a distinctly differential proteome between serum and plasma as well as normal and disease sera, providing a simple and convenient approach for serum and plasma differentiation and Hp phenotypes classification. Despite this, preliminary results cannot be used in early diagnosis of diseases at present, this study clearly demonstrates that BS4-DE would be a feasible and complete technology for protein biomarker screening in the future.

Our results suggest that BS4-DE system will be generally useful for protein complexomics research, especially for profiling of disease proteomics, owing to two major innovative techniques. One is native-tl-IEF/acidic-native-PAGE, which
expands the application range of BS4-DE for more than 1000 kinds of extremely basic proteins or complexes (the range of pI from about 8 to 11) separation with high resolution under mild condition. The other is NH₃H₂O-ultrasonic protein in-gel extractive strategy, which can achieve high efficiency recovery of protein extraction as well as avoid the influence of salt in following analysis. Therefore, it is a favorable reply for low-abundance proteins or complexes, ascribed to the limitlessness extraction and enrichment in theory. More importantly, this strategy achieves the seamless connection between part I and part II in a BS4-DE system. Notably, NH₃H₂O-ultrasonic protein in-gel extractive strategy may be valuable for “top-down” MS proteomics analysis, provided that the compatibility problem was solved. Furthermore, conservative protein-protein interactions can be easily confirmed in BS4-DE system proteomics analysis without the need of affinity purification, which could not be obtained from traditional 2-DE. Besides that, BS4-DE can be readily performed in every scientific or medical laboratory for differential protein complexome research. It is worthwhile to note that more than 4000 protein complexes could be theoretically separated by this system.

Many important questions remain to be addressed in subsequent studies. For example, it will be valuable to develop a basic-protein mixture for acidic-native-PAGE as marker. In addition, the detection of detailed heterogeneity and PTMs of protein complexes with enough cases based on BS4-DE system will be useful and necessary for revealing the correlations between protein complexes and diseases.

Acknowledgments—This study was approved by the biomedical ethics review committee at Institute of Basic Medical Sciences, Chinese Academy of Medical Sciences and School of Basic Medicine, Peking Union Medical College (reference No 002-2009).

* This work was supported by the National Natural Science Foundation of China under Grant No 21075137.
[S] This article contains supplemental Figs. S1 to S8 and Tables S1 to S4.

† To whom correspondence should be addressed: Institute of Basic Medical Sciences, Chinese Academy of Medical Sciences and School of Basic Medicine, Peking Union Medical College, 5 Dong Dan San Tiao, Beijing 100005, China. Tel.: +86-10-65296479; Fax: +86-10-65263815; E-mail: lizhili@ibms.pumc.edu.cn.

REFERENCES

1. Gavin, A. C., Böshe, M., Krause, R., Grandi, P., Marzioch, M., Bauer, A., Schultz, J., Rick, J. M., Michon, A. M., Cruciat, C. M., Remor, M., Hofert, C., Schelder, M., Brajenovic, M., Ruffner, H., Merino, A., Klein, K., Hudak, M., Dickson, D., Rudi, T., Gnau, V., Bauch, A., Bastuck, S., Huhse, B., Leutwein, C., Heurter, M. A., Copley, R. R., Edelmann, A., Querfurth, E., Rybin, V., Drewes, G., Raida, M., Bouwmeester, T., Bork, P., Seraphin, B., Küster, B., Neubauer, G., and Superti-Furga, G. (2002) Functional organization of the yeast proteome by systematic analysis of protein complexes. Nature 415, 141–147.

2. Ito, T., Tashiro, K., Muta, S., Ozawa, R., Chiba, T., Nishizawa, M., Yamamoto, K., Kuhara, S., and Sakaki, Y. (2000) Toward a protein–protein interaction map of the budding yeast: A comprehensive system to examine two-hybrid interactions in all possible combinations between the yeast proteins. Proc. Natl. Acad. Sci. U.S.A. 97, 1143–1147

3. Uetz, P., Giot, L., Cagney, G., Mansfield, T. A., Judson, R. S., Knight, J. R., Lockshon, D., Narayan, V., Sririnivasan, M., Pochart, P., Qureshi-Emili, A., Li, Y., Godwin, B., Conover, D., Kalbfleisch, T., Vijayadamodar, G., Yang, M., Johnston, M., Fields, S., and Rothberg, J. M. (2000) A comprehensive analysis of protein-protein interactions in Saccharomyces cerevisiae. Nature 403, 623–627

4. Venkatesan, K., Rual, J. F., Vazquez, A., Stezl, U., Lemmens, I., Hirozane-Kishikawa, T., Hao, T., Zenkner, M., Xin, X., Goh, K. I., Yildirim, M. A., Simonis, N., Heinzmann, K., Gebbre, F., Sahalie, J. M., Cevik, S., Simon, C., de Smet, A. S., Dann, E., Smolyar, A., Vinayagam, A., Yu, H., Szeto, D., Borick, H., Dricot, A., Klitgord, N., Murray, R. R., Lin, C., Lalovski, M., Timm, J., Rau, K., Boone, C., Braun, P., Cusick, M. E., Roth, F. P., Hill, D. E., Tavernier, J., Wanker, E. E., Barabási, A. L., and Vidal, M. (2009) An empirical framework for binary interactome mapping. Nat. Methods 6, 829–835

5. Stezl, U., Worm, U., Lalovski, M., Haenic, C., Brembeck, F. H., Goehler, H., Stroedicke, M., Zenkner, M., Schoenher, A., Koeppen, S., Timm, J., Mintzlaff, S., Abraham, C., Bock, N., Kietzmann, S., Goedeke, A., Toksőz, E., Droeg, A., Krobitsch, S., Korn, B., Brinchenwe, M., Lehrlach, H., and Wanker, E. E. (2005) A human protein-protein interaction network: A resource for annotating the proteome. Cell 122, 957–968

6. Rappsilber, J. (1999) Detection of protein-protein interactions by communoprecipitation and dimerization. Methods Enzymol. 254, 491–497

7. Ewing, R. M., Chu, P., Elisma, F., Li, H., Taylor, P., Clemie, S., McBroome-Cerajewski, L., Robinson, M. D., O’Connor, L., Li, M., Taylor, R., Dharsee, M., Ho, Y., Heilbut, A., Moore, L., Zhang, S., Omatsky, O., Buhak, Y. V., Ethier, M., Sheng, Y., Yasiscu, J., Abu Farha, M., Lambert, J. P., Duwel, H. S., Stewart, I., Kuehl, B., Hogue, K., Colwill, K., Gladwish, K., Misamore, R., Kinach, R., Adams, S. L., Moran, M. F., Morin, G. B., Topaloglu, T., and Figeys, D. (2007) Large-scale mapping of human protein-protein interactions by mass spectrometry. Mol. Syst. Biol. 3, 89

8. Wittig, I., Braun, H. P., and Schägger, H. (2006) Blue native PAGE. Nat. Protoc. 1, 418–428

9. Wittig, I., Karas, M., and Schägger, H. (2007) High resolution clear native electrophoresis for in-gel functional assays and fluorescence studies of membrane protein complexes. Mol. Cell Proteomics 6, 1215–1225

10. Wittig, I., and Schägger, H. (2005) Advantages and limitations of clear-native PAGE. Proteomics 5, 4338–4346

11. Gavin, P. D., Prescott, M., and Devenish, R. J. (2005) Yeast FIF0-ATP synthase complex interactions in vivo can occur in the absence of the dimer specific subunit e. J. Bioenerg. Biomembr. 37, 55–66

12. Pettler, J. B., Cai, Y., Sun, Q., Zabrouskov, V., Giacomelli, L., Rudella, A., Ytterberg, A. J., Rutschow, H., and van Wijk, J. K. (2008) The oligomeric stromal proteome of Arabidopsis thaliana chloroplasts. Mol. Cell. Proteomics 7, 114–133

13. Wittig, I., and Schägger, H. (2009) Native electrophoretic techniques to identify protein-protein interactions. Proteomics 9, 5214–5223

14. Manabe, T., Yamaguchi, N., Mukai, J., Hamada, O., and Tani, O. (2003) Detection of protein-protein interactions and a group of immunoglobulin G-associated minor proteins in human plasma by non-denaturing and denaturing two-dimensional gel electrophoresis. Proteomics 3, 832–846

15. Jin, Y., and Manabe, T. (2009) Performance of agarse IEF gels as the first dimension support for non-denaturing micro-2-DE in the separation of high-molecular-mass plasma proteins and protein complexes. Electrophoresis 30, 939–948

16. Manabe, T., and Jin, Y. (2010) Analysis of E. coli soluble proteins by non-denaturing micro 2-DE/3-DE and MALDI-MS-PMF. Electrophoresis 31, 2740–2748

17. Manabe, T., and Jin, Y. (2011) Performance of non-denaturing micro 2-DE followed by third-dimension SDS-PAGE in the analysis of Escherichia coli soluble proteins. Electrophoresis 32, 300–309

18. Wang, X., Chen, G., Liu, H., Zhao, Z., and Li, Z. (2010) Four-dimensional orthogonal electrophoresis system for screening protein complexes and protein-protein interactions combined with mass spectrometry. J. Proteome Res. 9, 5325–5334

19. Margolis, J., and Kenrick, K. G. (1969) Two-dimensional resolution of plasma proteins by combination of polyacrylamide disc and gradient gel electrophoresis. Nature 221, 1056–1057

20. Elasasser, S., Schmidt, M., and Finley, D. (2005) Characterization of the proteome using native gel electrophoresis. Methods Enzymol. 396, 353–363
