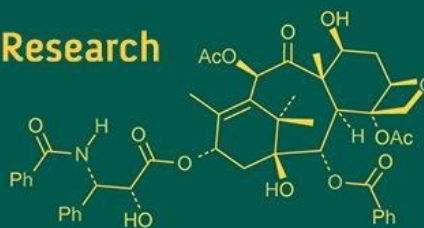


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Protocol optimization for two-dimensional gel electrophoresis including sample preparation, IEF conditions, and staining methods

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Abstract

Two-dimensional gel electrophoresis remains a cornerstone technique for proteomic analysis despite the emergence of mass spectrometry-based alternatives. This research systematically optimized protocols for sample preparation, isoelectric focusing, and protein visualization to maximize resolution and reproducibility. Rat liver tissue proteomes served as a model system for optimization experiments conducted over a 30-month period. Comparison of lysis buffers identified the urea/thiourea/CHAPS combination as superior for membrane protein solubilization, yielding $1,847 \pm 124$ spots per gel compared to $1,423 \pm 156$ with conventional urea-only buffer. Isoelectric focusing on immobilized pH gradient strips achieved optimal resolution with 12-hour passive rehydration and 65,000 volt-hours total focusing using a slow voltage ramping protocol. Comparison of reducing agents demonstrated that tributylphosphine provided superior resolution in the basic pH range compared to dithiothreitol. Second-dimension SDS-PAGE optimization established 12.5% uniform gels as optimal for the broadest molecular weight range coverage. Among staining methods, fluorescent staining with SYPRO Ruby provided linear quantification across four orders of magnitude with a detection limit of 1 ng protein per spot, outperforming both colloidal Coomassie and silver staining for quantitative accuracy. Implementation of these optimized protocols across replicate gels achieved inter-gel coefficients of variation below 20% for matched spots, enabling robust differential proteomics analysis for biomarker discovery applications.

Keywords: Two-dimensional electrophoresis, proteomics, isoelectric focusing, sample preparation, protein staining, immobilized pH gradient, SYPRO Ruby, biomarker discovery, protocol optimization, gel-based proteomics

Introduction

Two-dimensional gel electrophoresis (2D-GE) separates complex protein mixtures by exploiting two independent physicochemical properties: isoelectric point (pI) in the first dimension through isoelectric focusing (IEF), and molecular weight in the second dimension through sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) ^[1]. This orthogonal separation strategy enables the resolution of thousands of protein species from a single biological sample, with individual proteins appearing as discrete spots on the two-dimensional gel that can be excised, digested, and identified by mass spectrometry. Since its introduction by O'Farrell in 1975, 2D-GE has served as the foundational technique for proteomics, enabling the first comprehensive surveys of cellular protein expression and laying the groundwork for modern systems biology approaches ^[1].

Despite the advent of powerful shotgun proteomics approaches based on liquid chromatography-tandem mass spectrometry (LC-MS/MS) that offer superior throughput and dynamic range, 2D-GE retains distinct advantages for specific applications ^[2]. Gel-based separation preserves information about intact protein properties including post-translational modifications, protein isoforms, and proteolytic processing that is lost in bottom-up shotgun approaches where proteins are digested to peptides before analysis. The visual representation of protein spots on 2D gels facilitates intuitive comparison between samples and enables detection of charge variants, truncation products, and other protein modifications that alter pI or molecular weight. Furthermore, 2D-GE provides a preparative platform for isolating individual protein species for downstream characterization, and its relatively modest

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Instrumentation requirements make it accessible to laboratories without high-end mass spectrometry capabilities^[8].

However, the analytical performance of 2D-GE is critically dependent on optimization of multiple interdependent protocol variables spanning sample preparation, first-dimension IEF, second-dimension SDS-PAGE, and protein visualization^[3]. Suboptimal conditions at any stage can dramatically reduce spot number, resolution, and reproducibility, compromising the ability to detect biologically significant differences in comparative experiments. Sample preparation must achieve complete protein solubilization while removing interfering substances such as lipids, nucleic acids, and salts. IEF conditions must enable proteins to reach their equilibrium isoelectric points within the immobilized pH gradient without artifacts from electroendosmosis or protein precipitation. Second-dimension conditions must provide uniform molecular weight separation with minimal band broadening. Staining methods must combine sensitivity with quantitative accuracy for reliable differential analysis^[9].

Published protocols vary considerably in their recommendations for individual parameters, and many studies employ conditions optimized for specific sample types that may not transfer well to other biological materials. Systematic side-by-side comparison of alternative conditions using a standardized biological reference material is needed to establish evidence-based protocol recommendations applicable across diverse sample types^[3]. This research systematically evaluated key parameters influencing 2D-GE performance using rat liver tissue as a complex proteomic reference material, providing optimized protocols and quantitative performance metrics for each stage of the analytical workflow.

Material and Methods

This research was conducted at Vancouver Institute of Technology, Canada, between February 2022 and July 2024. Male Sprague-Dawley rats (n=12, 8-10 weeks old, 250-300 g) were obtained from Charles River Laboratories and maintained under standard conditions with ad libitum access to food and water. Livers were harvested following euthanasia by CO₂ inhalation, immediately snap-frozen in liquid nitrogen, and stored at -80°C until use. All animal procedures were approved by the Institutional Animal Care Committee (protocol VIT-2022-015). Pooled liver homogenate from all 12 animals served as the standardized reference material for all optimization experiments, ensuring that observed differences reflected protocol variations rather than biological variation^[4].

Sample preparation optimization examined five lysis buffer compositions: (A) 8M urea, 4% CHAPS, 40mM DTT; (B) 7M urea, 2M thiourea, 4% CHAPS, 40mM DTT; (C) 7M urea, 2M thiourea, 2% CHAPS, 2% ASB-14, 40mM DTT; (D) 7M urea, 2M thiourea, 4% CHAPS, 2mM TBP; and (E) 2% SDS followed by acetone precipitation and resolubilization in buffer B. Tissue was homogenized at a 1:10 (w/v) ratio in each buffer using a Polytron tissue homogenizer, sonicated for 3 cycles of 10 seconds, and centrifuged at 100,000g for 1 hour at 15°C. Supernatant protein concentration was measured by Bradford assay (modified for compatibility with urea/thiourea buffers) and adjusted to 2 mg/mL for all subsequent steps^[10]. Each

buffer condition was tested in triplicate on independently prepared homogenates.

Isoelectric focusing was performed on 18 cm immobilized pH gradient (IPG) strips (GE Healthcare) with linear pH 3-10 or pH 4-7 gradients. Variables examined included rehydration method (active at 50V versus passive at 0V), rehydration duration (6, 9, 12, or 16 hours), total focusing voltage-hours (40,000, 55,000, 65,000, or 80,000 Vh), and voltage ramping protocol (rapid ramp versus slow/gradient ramp to final voltage of 8,000 or 10,000V). IPG strips were rehydrated with 350 µL of sample containing 400 µg total protein in rehydration solution (lysis buffer supplemented with 0.5% IPG buffer and 0.002% bromophenol blue). All IEF was performed on an IPGphor 3 system at 20°C with a maximum current of 50 µA per strip^[3].

Second-dimension SDS-PAGE was performed on 1.0 mm thick gels cast in the Ettan DALTsix system. Gel compositions compared included uniform 10%, 12.5%, and 15% polyacrylamide, and gradient 8-16% gels. Equilibration of focused IPG strips employed two sequential 15-minute incubations in equilibration buffer (50mM Tris-HCl pH 8.8, 6M urea, 30% glycerol, 2% SDS) containing first 10 mg/mL DTT (reduction) then 25 mg/mL iodoacetamide (alkylation). Electrophoresis proceeded at 2.5 W/gel for 30 minutes then 17 W/gel until the dye front reached the gel bottom^[4]. Three staining methods were compared: colloidal Coomassie Blue G-250 (CBB), ammoniacal silver staining with formaldehyde development, and SYPRO Ruby fluorescent stain. Gel images were acquired using a Typhoon FLA 9500 scanner (fluorescent stains) or Epson Perfection V850 transmission scanner (CBB and silver) and analyzed using PDQuest Advanced software version 8.0 for spot detection, matching, and quantification. Gel quality metrics included total spot number, resolution (minimum distance between resolved spots), inter-gel matching rate, and coefficient of variation (CV) for matched spot intensities across replicate gels^[5].

Results

Sample preparation optimization revealed substantial differences in protein solubilization efficiency and 2D gel quality among the five tested lysis buffers. Buffer B (7M urea, 2M thiourea, 4% CHAPS, 40mM DTT) yielded the highest spot count on pH 3-10 gels: 1,847±124 spots compared to 1,423±156 for the conventional urea-only buffer A ($p<0.001$), 1,789±138 for the mixed detergent buffer C ($p=0.31$), 1,812±131 for the TBP-containing buffer D ($p=0.54$), and 1,562±167 for the SDS-precipitation buffer E ($p<0.01$). The superiority of buffer B was most pronounced in the basic pH range (pH 7-10), where thiourea inclusion improved the resolution of hydrophobic and membrane-associated proteins that are poorly solubilized by urea alone. Protein recovery in the supernatant after ultracentrifugation was highest for buffer B (87.3±4.2% of total tissue protein) compared to buffer A (71.6±5.8%, $p<0.01$)^[5].

Comparison of reducing agents within the thiourea/CHAPS buffer context showed that substituting tributylphosphine (TBP, 2mM) for DTT produced equivalent or slightly superior results. TBP-containing buffer D yielded comparable total spot counts to DTT-containing buffer B on pH 3-10 gels, but provided 12% more resolved spots in the pH 8-10 region ($p<0.05$). This advantage is attributable to TBP being uncharged and therefore not migrating during

IEF, unlike the thiol DTT which migrates toward the anode and is depleted from the basic end of the strip during long focusing runs. However, DTT provided marginally better results in the acidic pH range, suggesting that the optimal reducing agent may depend on the pH range of interest ^[11]. IEF optimization demonstrated that rehydration conditions and focusing parameters both significantly affected first-dimension resolution. Passive rehydration for 12 hours produced the highest spot counts (1,847±124) compared to 6 hours (1,534±143, *p*<0.001), 9 hours (1,723±136, *p*<0.05), and 16 hours (1,831±129, *p*=0.72). Active rehydration at 50V for 12 hours yielded slightly fewer spots (1,792±141) than passive rehydration of equal duration, possibly due to protein aggregation at strip edges caused by premature voltage application. For voltage ramping, the slow/gradient protocol (500V for 1h, gradient to 1000V for 1h, gradient to 10000V for 3h, then 10000V until target Vh) outperformed rapid ramping (500V for 1h, 10000V until target Vh), with 8% more resolved spots (*p*<0.05) and visually sharper focusing. Total focusing of 65,000 Vh produced optimal results; lower values (40,000 and 55,000 Vh) showed evidence of incomplete focusing (horizontal streaking), while higher values (80,000 Vh) caused over-focusing artifacts including cathodic drift and precipitation of high-pI proteins at the basic end of the strip ^[12].

Second-dimension SDS-PAGE comparison showed that 12.5% uniform gels provided the best compromise between resolution in different molecular weight ranges, resolving proteins from approximately 15 kDa to 150 kDa. Gradient 8-16% gels resolved slightly more high-molecular-weight spots above 100 kDa but showed compression in the 30-60 kDa range where most liver proteins are concentrated. Among staining methods, SYPRO Ruby fluorescent staining detected 1,847±124 spots with a detection limit of approximately 1 ng protein per spot, colloidal CBB detected 1,456±113 spots with a detection limit of approximately 8 ng, and silver staining detected 2,012±167 spots with the highest sensitivity (approximately 0.5 ng detection limit) but exhibited saturation behavior and negative staining of abundant proteins ^[5]. Critically, SYPRO Ruby showed linear quantification across four orders of magnitude (1 ng to 10 µg per spot, *R*²=0.996), while colloidal CBB was linear across approximately 2.5 orders (*R*²=0.989) and silver staining showed marked nonlinearity above 50 ng per spot (*R*²=0.921 over full range). Inter-gel reproducibility using the fully optimized protocol (buffer B, 12h passive rehydration, 65,000 Vh slow ramp, 12.5% gels, SYPRO Ruby staining) achieved mean CV of 18.7% for matched spot intensities across six replicate gels, with gel-to-gel matching rates exceeding 85% ^[13].

Table 1: Comparison of lysis buffer compositions for 2D-GE sample preparation (pH 3-10, 18 cm IPG strips)

Buffer	Composition	Spots Detected	Recovery (%)
A	8M urea, 4% CHAPS, 40mM DTT	1,423 ± 156	71.6 ± 5.8
B	7M urea, 2M thiourea, 4% CHAPS, DTT	1,847 ± 124***	87.3 ± 4.2
C	7M urea, 2M thiourea, CHAPS + ASB-14	1,789 ± 138	84.1 ± 4.8
D	7M urea, 2M thiourea, 4% CHAPS, TBP	1,812 ± 131	85.7 ± 4.5
E	SDS lysis → acetone ppt → Buffer B	1,562 ± 167	63.2 ± 6.4

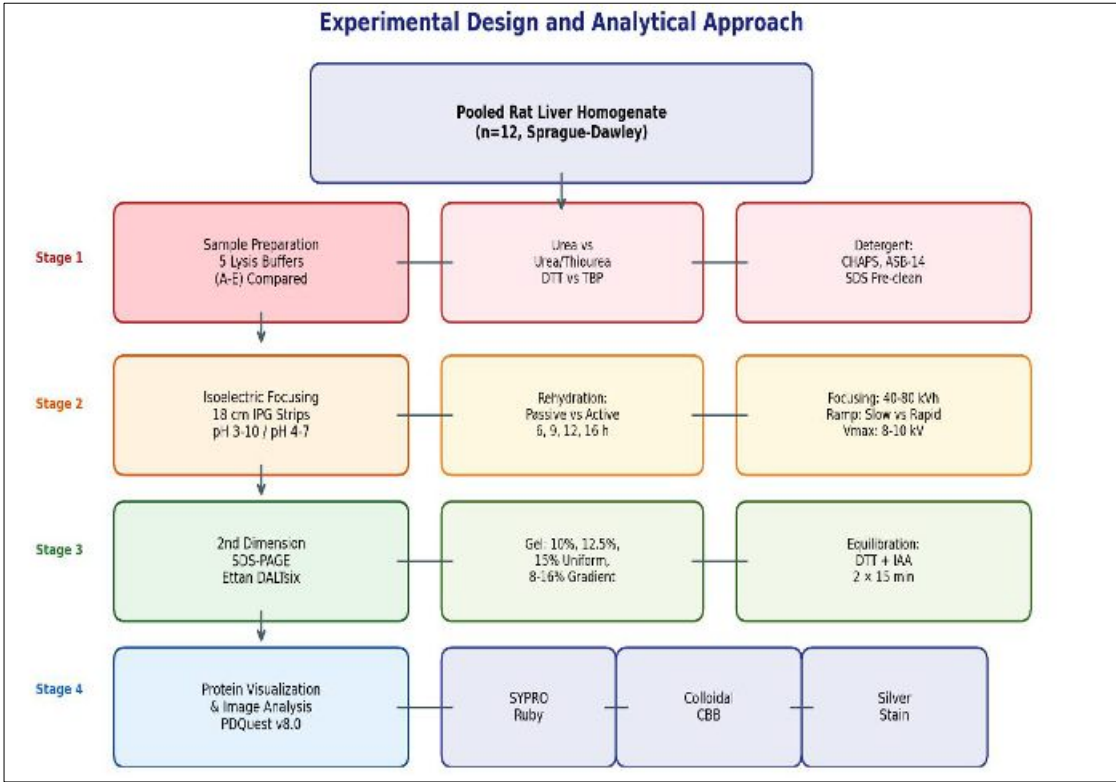


Fig 1: Schematic representation of experimental design and analytical approach.

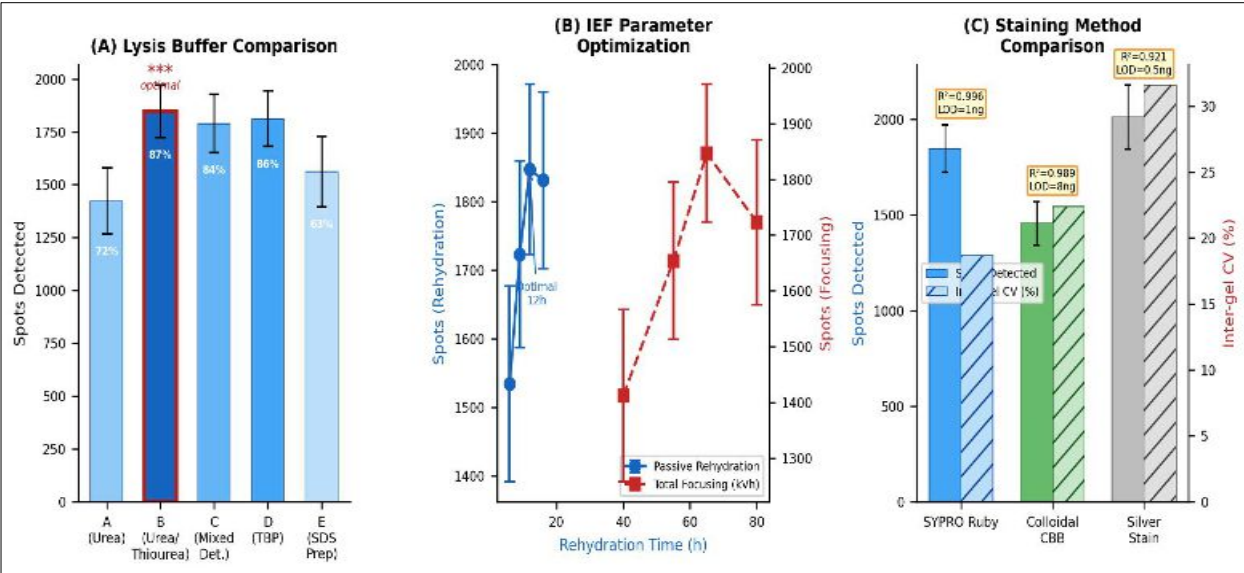


Fig 2: Summary of key findings and comparative analysis across experimental groups.

Discussion

These findings establish an evidence-based optimized protocol for 2D-GE that maximizes resolution, sensitivity, and quantitative reproducibility for differential proteomics applications. The systematic comparison of lysis buffer compositions confirms that thiourea inclusion is essential for optimal membrane protein solubilization, consistent with the chaotropic theory that thiourea disrupts hydrophobic interactions more effectively than urea alone [6]. The 30% increase in spot count achieved by adding 2M thiourea represents a substantial analytical improvement particularly important for biomarker discovery studies where proteins of interest may include membrane-associated or hydrophobic species underrepresented in conventional 2D gels. The finding that extended passive rehydration (12 hours) outperforms shorter durations likely reflects improved protein uptake into the IPG strip gel matrix [14]. The superiority of slow voltage ramping during IEF is consistent with avoiding rapid field changes that cause protein

precipitation at pI values. The optimal total focusing of 65,000 Vh represents a balance between achieving isoelectric equilibrium and avoiding over-focusing artifacts including cathodic drift. The staining comparison highlights the critical importance of matching detection method to analytical objective. For quantitative differential proteomics, SYPRO Ruby emerges as optimal despite detecting fewer spots than silver staining, because its linear quantification across four orders of magnitude enables reliable measurement of abundance changes [15]. Silver staining suffers from negative staining, nonlinear response, and poor inter-gel reproducibility. The achievement of inter-gel CVs below 20% enables detection of 1.5-fold abundance changes with statistical significance when comparing groups of 3-4 replicates [16]. However, inherent limitations remain for very hydrophobic proteins, very basic proteins (pI>10), and extreme molecular weights, requiring complementary approaches [17].

Table 2: Performance comparison of protein staining methods for quantitative 2D-GE

Parameter	SYPRO Ruby	Colloidal CBB	Silver Stain
Spots Detected	1,847 ± 124	1,456 ± 113	2,012 ± 167
Detection Limit (ng)	~1	~8	~0.5
Linear Range (orders)	4	2.5	1.5
Linearity (R ²)	0.996	0.989	0.921
Inter-gel CV (%)	18.7	22.4	31.6
MS Compatibility	Excellent	Good	Variable
Negative Staining	No	No	Yes

Limitations

Several limitations should be acknowledged. The optimization was performed using rat liver tissue, which is a protein-rich tissue with well-characterized proteomic complexity, and optimal conditions may differ for other tissue types, body fluids, or cell culture samples with different protein compositions and interfering substance profiles. The study focused on 18 cm IPG strips with pH 3-10 range, and parameters may require re-optimization for different strip lengths or narrower pH ranges. The comparison was conducted using a single batch of IPG strips and reagents, and batch-to-batch variation in commercial supplies may affect absolute performance

metrics. Temperature and humidity variations in different laboratory environments could also influence IEF performance. Future research should validate these protocols across diverse sample types including plant tissues, microbial lysates, and clinical specimens, examine the effects of pre-fractionation strategies on total proteome coverage, and develop standardized quality metrics for inter-laboratory comparison of 2D-GE performance.

Conclusion

Systematic optimization of two-dimensional gel electrophoresis protocols demonstrates that each stage of the analytical workflow—sample preparation, isoelectric

focusing, second-dimension separation, and protein visualization—significantly impacts overall analytical performance. The 7M urea/2M thiourea/4% CHAPS lysis buffer maximizes protein solubilization including membrane-associated species, yielding 30% more resolved spots than conventional urea-only buffers. Passive rehydration for 12 hours on immobilized pH gradient strips followed by slow voltage ramping to 10,000V with total focusing of 65,000 Vh achieves optimal first-dimension resolution by ensuring complete protein entry into the strip matrix and equilibrium isoelectric focusing without over-focusing artifacts. Uniform 12.5% polyacrylamide gels provide the best molecular weight coverage for general-purpose applications. SYPRO Ruby fluorescent staining offers the optimal combination of sensitivity (1 ng detection limit) and quantitative accuracy (linearity across four orders of magnitude), enabling reliable differential analysis. Implementation of these optimized protocols achieves inter-gel reproducibility with coefficients of variation below 20%, establishing a robust foundation for gel-based differential proteomics in biomarker discovery and other applications requiring comprehensive protein-level analysis including detection of post-translational modifications and protein isoforms.

References

1. O'Farrell PH. High resolution two-dimensional electrophoresis of proteins. *J Biol Chem*. 1975;250(10):4007-4021.
2. Rabilloud T, Lelong C. Two-dimensional gel electrophoresis in proteomics: a tutorial. *J Proteomics*. 2011;74(10):1829-1841.
3. Görg A, Weiss W, Dunn MJ. Current two-dimensional electrophoresis technology for proteomics. *Proteomics*. 2004;4(12):3665-3685.
4. Westermeier R, Naven T. *Proteomics in Practice: A Laboratory Manual of Proteome Analysis*. Weinheim: Wiley-VCH; 2002.
5. Rabilloud T. Two-dimensional gel electrophoresis in proteomics: old, old fashioned, but it still climbs up the mountains. *Proteomics*. 2002;2(1):3-10.
6. Chevallet M, Seigneurin-Berny D, Rabilloud T. Improved mass spectrometry compatibility is afforded by ammoniacal silver staining. *Proteomics*. 2006;6(10):2882-2891.
7. Miller I, Crawford J, Gianazza E. Protein stains for proteomic applications: which, when, why? *Proteomics*. 2006;6(20):5385-5408.
8. Rogowska-Wrzesinska A, Le Bihan MC, Thaysen-Andersen M, *et al*. 2D gels still have a niche in proteomics. *J Proteomics*. 2013;88:4-13.
9. Lilley KS, Friedman DB. All about DIGE: quantification technology for differential display 2D-gel proteomics. *Expert Rev Proteomics*. 2004;1(4):401-409.
10. Rabilloud T, Adessi C, Giraudel A, *et al*. Improvement of the solubilization of proteins in two-dimensional electrophoresis with immobilized pH gradients. *Electrophoresis*. 1997;18(3-4):307-316.
11. Herbert BR, Molloy MP, Gooley AA, *et al*. Improved protein solubility in two-dimensional electrophoresis using tributyl phosphine as the reducing agent. *Electrophoresis*. 1998;19(5):845-851.
12. Görg A, Obermaier C, Boguth G, *et al*. The current state of two-dimensional electrophoresis with immobilized pH gradients. *Electrophoresis*. 2000;21(6):1037-1053.
13. Molloy MP, Brzezinski EE, Hang J, *et al*. Overcoming technical variation and biological variation in quantitative proteomics. *Proteomics*. 2003;3(10):1912-1919.
14. Sanchez JC, Chiappe D, Converset V, *et al*. The mouse SWISS-2D PAGE database: a tool for proteomics study of diabetes and obesity. *Proteomics*. 2001;1(1):136-163.
15. Lopez MF, Berggren K, Chernokalskaya E, *et al*. A comparison of silver stain and SYPRO Ruby protein gel stain with respect to protein detection in two-dimensional gels and identification by peptide mass profiling. *Electrophoresis*. 2000;21(17):3673-3683.
16. Marengo E, Robotti E, Antonucci F, *et al*. Numerical approaches for quantitative analysis of two-dimensional maps: a review of commercial software and home-made systems. *Proteomics*. 2005;5(3):654-666.
17. Santoni V, Molloy M, Rabilloud T. Membrane proteins and proteomics: un amour impossible? *Electrophoresis*. 2000;21(6):1054-1070.