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Two-Dimensional Polyacrylamide Gel Electrophoresis – A Practical Perspective

Sameh Magdeldin[1,2], Ying Zhang[1], Bo Xu[1],
Yutaka Yoshida[1] and Tadashi Yamamoto[1]
[1]*Department of Structural Pathology, Institute of Nephrology, Graduate School of Medical and Dental Sciences, Niigata University,*
[2]*Department of Physiology, Faculty of Veterinary Medicine, Suez Canal University, Ismailia,*
[1]*Japan*
[2]*Egypt*

1. Introduction

Two- dimensional electrophoresis, abbreviated as 2-DE, is one of the most powerful and common tools for separation and fractionation of complex protein mixture extracted from tissues, cells, and other biological specimens. It is an orthogonal technique that allows separation of thousands of proteins in one gel and in a two tandem electrophoretic steps where a major proportion of proteins can be resolved for further analysis. Since the pioneer development and modification of high resolution 2-DE by (O'Farrell, 1975), the usage of this technique is increasing and proven to be effective. In fact, the modification set by O'Farrell empowered this technique to resolve up to 5000 spots in a uniform distribution and on a single gel. Protein spot can be seen as little as one disintegration per min by autoradiology or recent quantitative dye. This high reproducibility of separation allows comparative matching for detection of dysregulated proteins.

The emerging date of 2-DE started since 1969 to 1974. At least several attempt to develop 2-DE methods were described (Macko & Stegemann, 1969; Margolis & Kenrick, 1969; Mets & Bogorad, 1974; Orrick et al, 1973). However, in terms of resolved proteins spots and reproducibility, the procedure of (Kaltschmidt & Wittmann, 1970) has been widely used. Although, at that time, the technique has been suffering from limited resolution and reproducibility, it has been used by many researchers for investigating ribosomal assembly and structure. Few years later, noticed improvements has been added on by (O'Farrell, 1975).

O'Farrell optimized 2-DE on the basis that each separation must be done in independent parameter, otherwise protein will be distributed across a diagonal rather than across the entire surface of the gel. To perform this optimization, the first electrophoretic separation referred as 1D or Isoelectric focusing (IEF) and discontinuous sodium dodecyl sulphate (SDS) gel system (Laemmli, 1970) was chosen because its high resolution power and ability to separate protein according to its isoelectric point. In his original manuscript, the first dimension separation was performed in carrier ampholyte- containing polyacrylamide gel cast in narrow tubes to provide a pH gradient atmosphere for protein migration.

Since these procedures aim principally to fractionate proteins, denaturation, reduction and unfolding is required. Hence, denaturant and reductant agents should be added along with the experimental procedures.

As mentioned earlier, the principal of protein separation in 2-DE is performed into two major steps; first dimension and second dimension. In the first dimension, proteins are resolved in according to their isoelectric point (PI) and separated in a pH gradient into a sharp band. Using different approaches either immobilized gradient electrophoresis (IPEG), isoelectric focusing (IEF), or non-equilibrium pH gradient electrophoresis (NEPHGE). In the second dimension, proteins are separated according to their molecular weight using SDS Laemmli system. Both separations are carried out in polyacrylamide gel. Because it is unlikely that different molecules may have the same physicochemical properties (PI and Mw), proteins are more effectively separated by 2-DE rather than 1D-SDS PAGE. An outstanding feature of 2-DE is that the resolution obtained during the first dimensional separation is not lost when the IEF gel is joined to the SDS- PAGE gel in the 2nd electrophoresis(Anderson et al, 2001).

Tools and equipments for 2-DE are readily available with reasonable price. The recent availability of high quality IPGs strips made in multitude range of pH and lengths goes a long way towards ensuring reproducibility and better performance of the first dimensional electrophoresis. Similarly, the availability of pre- casted gels and casting devices in different sizes enabled laboratory investigations to produce more effective and reproducible results. In addition, facilitated interlaboratory comparison and collaboration.

The introduction of immobilized pH gradient reagent by Gorg and colleagues significantly improved the resolution of the first dimension (Gorg et al, 1999; Gorg et al, 1988c; Gorg et al, 1983). They replaced the carrier ampholate- generated pH gradient with immobilized pH gradient and the tube gels were replaced gels supported with plastic backing. This change did not only improve the separation performance but also made the technical procedures easier for researchers.

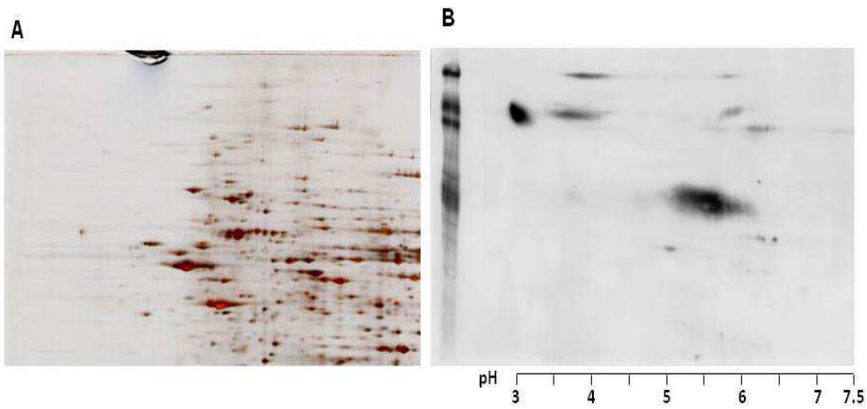


Fig. 1. Glomerular protein resolved on 8 cm IGP strip and subjected for 2-DE. A) Silver stained gel and B) the gel was blotted on PVDF membrane and subjected to western blotting for detecting phosphorylated proteins using P- Tyr- 100 antibody (Zhang et al, 2010).

To date, 2-DE technique has been one of the driving forces in the development of proteomics and protein analysis. It compromise an initial step for further analysis of these differentially

regulated protein spots by mass spectrometry and western blotting and finally the use of 2-DE has successfully pinpointed, in many cases, a defected disease- related protein that ,no doubt, facilitated biomarker discovery.

2. Basic principles of 2-DE

2.1 First blossom of two-dimensional gel electrophoresis

SDS electrophoresis (1-D electrophoresis) was introduced in early 70s (Laemmli, 1970) and has been widely used in many of life science fields to profile a complex or less complex protein mixture. It is now widely admitted that this method provide the resolution far from being sufficient to separate protein components, and used most frequently in combination with immunoblotting or overlay analysis, and in pre-fractionation step to reduce complexity prior to analysis with mass spectrometers.

The seminal emergence of two-dimensional gel electrophoresis (2-D electrophoresis) technique (Klose, 1975; O'Farrell, 1975) deeply impressed many researchers with its strong resolution power, which was able to separate metabolically isotope-labeled proteins into a maximum of 5,000 evenly distributed, discrete spots on a standard-sized slab gel. This technique used the two principal physicochemical properties of proteins, i.e., isoelectric point (pI) and molecular weight (Mw), and implicated isoelectric focusing (IEF) in polyacrylamide gel in the first dimension and SDS slab polyacrylamide gel electrophoresis (SDS-PAGE) in the second dimension.

2.1.1 Second booming of 2-D electrophoresis

The 2-D electrophoresis was extensively applied to biochemistry, cell biology and clinical biology in the late 70s and early 80s. However, it is long and complicated procedure, the low reproducibility and the lack of readily available protein identification tools had hampered its spreading as a common analytical tool until the take-off of proteomics, which used mass spectrometry, first mass fingerprinting with Time-of-Flight mass spectrometer and later shotgun analysis with LC-tandem mass spectrometer in late 90s and early 2000s. At that time, most of researchers were confident that this combination could be able to resolve complete proteomes, but it soon appeared that this would not be the case.

In this review, we overviewed the principles of 2-D electrophoresis analysis in modern proteomics, adverting to the limits and the advantages of 2-D electrophoresis, and put special emphasis on the strength of this classical, well characterized, but still useful technique for protein analysis, by highlighting its particular advantages which could not be replaceable by another proteomic approaches such as shotgun LC-tandem mass spectrometry.

2.2 Limitations of 2D electrophoresis

2.2.1 Low reproducibility

In the original and still employed method of the first dimension IEF, carrier ampholytes are used to build pH gradient. However, carrier ampholytes-based pH gradient made in soft unsupported tube gels (typically 4 % acrylamide) are not really stable, depending on lot of ampholytes, and prone to cathodic drift (a progressive loss of basic proteins during long

running of electrofocusing under electric field), leading to low reproducibility and requiring of careful control of application of electric field. The key development in improving reproducibility of 2-D electrophoresis was the introduction of immobilized pH gradients (IPG) that almost replaced carrier ampholyte-based pH gradient in tube gel (Gorg et al, 1988a; Gorg et al, 1988b). Although procedure for casting of IPG gels was described in details (Bossi et al, 1988; Gianazza, 1999), precast IPG gels with wide or various narrow range of pH are now commercially available (but more expensive). IPG gels with narrow pH gradients are most helpful to separate closely arrayed spots for accurate analysis of protein spots of interest.

2.2.2 Difficulty in separation of hydrophobic proteins

With the advent of mass spectrometry for identification of protein spots on 2-D gels, a plethora of analyses have been performed, and it soon became obvious that the same types of proteins were found again and again (Petrak et al, 2008), and the same types of proteins were always missing. The former could be explained by low dynamic range of 2-D electrophoresis, and the later by difficulty in separation of hydrophobic proteins (Corthals et al, 2000; Wilkins et al, 1998).

The difficulty in separation of hydrophobic proteins is clearly confined to the IEF dimension and to the chemical conditions at this step such as low ionic strength and no ionic detergents. Although many efforts have been done to aim at better solubilization of membrane proteins by changing chaotropes and detergents, it is reasonable to say that the problem is largely unsolved and remains a built-in problem (Rabilloud et al, 1997).

2.2.3 Narrow dynamic range of 2D electrophoresis

The problem of low dynamic range of 2-D electrophoresis is acute. With the availability of highly sensitive silver-staining method (Chevallet et al, 2008) and a variety of fluorescence dye with a wider dynamic range such as SYPRO-Ruby, protein spots separated on 2-D gels could be visualized to detection level below 1 ng. This inevitably leads to loss of low-abundance proteins, which are present under the detection level, and not subjected to analysis by mass spectrometry. The limited detection sensitivity of 2-D electrophoresis does not cope with the actual dynamic range of protein concentration in cell and tissue extracts, and biological fluids. Loading of a much more proteins and using of giant 2-D gels could be one solution, but possibly will lead to production of gel images crowded with not well-separated spots, since 2-D electrophoresis resolves many modified forms of high-abundance proteins, which will occupy separation space of 2-D gels.

2.3 Advantages and strengths of 2D electrophoresis

2.3.1 Robustness

Although low reproducibility had been evident in the early stage of development of 2-D electrophoresis, inter-experiments and even inter-laboratory variability has been much improved with standardization of 2-D electrophoresis procedures and visualization of protein spots by staining with recent staining procedures using fully mass spectrometry-compatible fluorescent dyes such as SYPRO-Ruby and Deep Purple. The most critical variable now does not reside in 2-D electrophoresis itself, but in upstream of 2-D electrophoresis workflow, i.e. sample preparation.

The 2-D electrophoresis, especially IEF in the first dimension, is very sensitive to many interfering compounds including lipids, nucleic acids, and small ionic molecules. These contaminants can be eliminated by additional steps such as organic solvent precipitation, dialysis or ultrafiltration, and nuclease treatment. It should be noted that these additional steps possible results in loss of some proteins, and that sample solubilization depends on the type of sample, and therefore the composition of the lysis solution should be optimized to each sample type. Dithiothreitol (DTT), the most commonly used reducing agent in 2-D electrophoresis, is charged and is eliminated out of the pH gradient during IEF, resulting in decreased solubility of some proteins. It has been reported that tributyl phosphine (TBP) could be more effective for protein solubilization than traditional reducing agents such as 2-mercaptoethanol and DTT (Rabilloud et al, 1997), although it is rather unstable in aqueous solution.

Staining procedure of 2-D gels is critical for quantitative analysis of 2-D gels. Classical silver-staining method is sensitive to detect as low as 0.3 ng protein spot but is not compatible to mass spectrometry due to use of formaldehyde or glutaraldehyde in the fixing and sensitization step, which introduces crosslinking of lysine residue within protein chain affecting MS analysis by hampering trypsin digestion. To overcome this drawback, several modifications of the silver nitrate staining have been developed (Chevallet et al, 2008; Shevchenko et al, 1996). The drawback of silver staining is its low dynamic range: the linear dynamic range of silver stain is restricted to approximately a tenfold, which makes this method unsuitable for quantitative analysis. In contrast, highly sensitive fluorescence dyes such as SYPRO-Ruby with wide range of linearity over several orders of magnitude provide accurate quantification of both high and low abundance proteins (Lopez et al, 2000). Protein profiles can be scanned to create gel images and analyzed using appropriate software to find differentially expressed proteins.

Software for analysis of 2-D gel images detects spots in individual 2-D gel images, matches spots between 2-D gel images under comparison, normalizes volume of defined spot area in individual gel images, and performs statistical comparison. The software packages include Delta2D, Image Master, Melanie, PDQuest, Progenesis, and REDFIN. It is noted that there are still problems associated with the quantitative analysis, especially in less defined, less-separated spots.

Fluorescence technology also offers multiplexed detection of three 2-D gel images from one 2-D gel using three succinimidyl ester derivatives of the fluorescent cyanin dyes, Cy2, Cy3 and Cy5, which exhibit different excitation and emission spectra. Briefly two samples to be compared are pre-labeled in vitro by Cy3 and Cy5, respectively, or vice versa, and composite sample (internal standard) prepared by mixing equal volume of the two samples is labeled with Cy2. These three samples are equally mixed and run within the same 2-D gel. The three 2-D gel images are separately taken using a confocal laser scanner, which are analyzed by computer-assisted overlay method. This technology, termed 2D differential gel electrophoresis (2D DIGE), allows a highly reproducible, quantitative analysis (Castillejo et al, 2011; Heywood et al, 2011; Wang et al, 2011).

2.3.2 Top-down proteomics or analysis of complete proteins

The major advantage of 2-D electrophoresis that should be emphasized is its unique ability to separate complete native proteins with all their modifications. The uncertainty pertinent

to identification of proteins in the shotgun tandem mass spectrometer analysis could be significantly decreased if the physicochemical properties (pI and Mw) are provided by 2-D electrophoresis. One of key application of this feature is analysis of post-translational and chemical modifications of proteins. 2-D electrophoresis provides modified proteins as a chain of spots with different pI. One best example is probably phosphorylation: 2-D gels could show their ability to separate and quantify the various phosphorylated forms of a protein of interest. Antibody-based analysis (i.e. immunoblotting) and mass spectrometry may contribute to detailed analysis of phosphorylated variants of a protein.

As mentioned above, the intactness of proteins separated on 2-D gels also allow us to use common immunological identification. 2-D gels can be directly used for immunoblotting analysis. This analysis was classical, but still has very valuable application: detection of modified amino acids such as phosphotyrosine and nitrosyltyrosine, and detection of other oxidatative stress-induced modifications such as citrullination, protein carbonylation, hydroxynonenal adducts, or changes in the thiol oxidation (Rabilloud et al, 2010).

Another application of this key feature of 2-D electrophoresis is to analysis of degradation products of a protein or proteins under certain physiological or pathological conditions. This type of analysis could be very difficult in the shotgun tandem mass spectrometric analysis and only possible in 2-D electrophoresis.

2.4 Concluding remarks

Two-dimensional gel electrophoresis has been used for long years in proteomics. With advent of mass spectrometry for identification of protein spots on 2-D gels, 2-D electrophoresis and mass spectrometry have been one of core technology in proteomics. Accumulation of data and experiences in the gel-based proteomics disclosed the built-in limitations in 2-D electrophoresis; low reproducibility, difficulty in separation of hydrophobic proteins, and narrow dynamic range. However, the strength of 2-D electrophoresis has been also recognized: improved robustness and reproducibility, unique ability to analyze complete native proteins with all modifications. Taken together, it is reasonable to mention that 2-D electrophoresis will produce best or better results when samples with a limited range of protein expression, i.e. samples with low complexity such as an organelle, subcellular fractions, protein complex, cerebrospinal fluid are analyzed.

3. Outlines on the types and current availability of 2-DE

As noted earlier, 2D- PAGE consists of a stepwise combination of two electrophoretic patterns; isoelectric focusing (IEF) followed by SDS-PAGE. The fractionation of proteins relays on protein isoelectric point for the first dimension and molecular weight for the second dimension.

The first dimension separation comprises [Conventional isoelectric focusing (IEF), non-equilibrium pH gel electrophoresis (NEPHGE), or immobilized pH gradient (IPG)].

The second dimension electrophoresis (SDS-PAGE).

3.1 First dimension electrophoresis

The first dimension electrophoresis can be carried out by a carrier ampholyte pH gradient (ionic substance that is capable of either reacting as an acid or as a base) or recently, it has

been replaced with a well-defined immobilized pH gradient which increased the separation resolution and enabled a high protein load capacity with less labour effort (Gorg et al, 1983). In the old fashion procedure of ampholyte, proteins migrate within this carrier ampholyte in a solution media until it reaches the equilibrium state (when the net charge of protein molecule equals zero). On the other hand, immobilized pH gradient strip is an integrated part of polyacrylamide gel matrix fixed on a plastic strip. In this method, copolymerization of a set of non amphoteric buffers with various chemical characters are included (Bjellqvist et al, 1982). Commercially, a wide range of length and pI are available; for example, a strip length from 7 to 24 and a pI ranged from 3-10, 4-6, 5-7, 8-9 are produced from many commercial companies.

Below is an example for IPG strips [Immobiline dry strip] with their ranges and their relative focusing power. (Adopted from GE healthcare).

	IPG Strips					IPG Buffers							
	Strip length					pH range							
	24 cm	18 cm	13 cm	11 cm	7 cm	3.5-5.0	5.5-6.7	4-7	6-11	7-11 NL	3-10 NL	3-10	3-11 NL
Narraw													
3.5-4.5	×					•							
5.3-6.5	×	×	×	×	×		•						
6.2-7.5	×	×	×	×	×				•				
Medium													
3-5.6 NL	×	×	×	×	×	•							
3-7 NL	×							•					
4-7	×	×	×	×	×			•					
6-9	×	×							•				
6-11		×	×	×	×				•				
7-11 NL	×	×	×	×	×					•			
Wide													
3-10	×	×	×	×	×							•	
3-11 NL	×	×	×	×	×								•
3-10 NL	×	×	×		×						•		

Fig. 2. Wide ranges of IPG steps with different PI are available commercially.

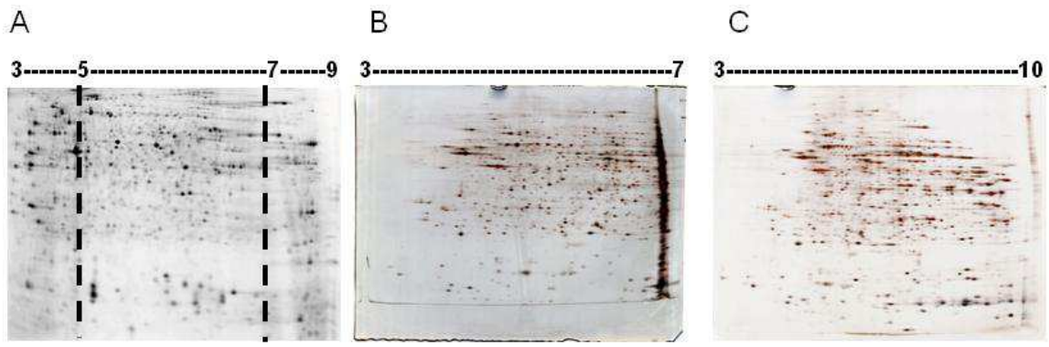


Fig. 3. Different examples of 2- DE gels. A) The usage of non-liner (NL) IPG strips fractionates mainly proteins with PI 5-7. B) 8 cm 2- DE gel using IPG strips with PI [3-7]. C) 18 cm 2- DE gel with a broad IPG strip PI [3-10].

Another available type of IPG strips produced by Amersham is non-linear (NL) series. For instance, 3-10 NL,18 or 24 cm which enables focusing of the majority of neutral proteins with PI from 5-7 and with a smaller focusing length to the extreme acidic (3-5) and basic (8-

10) proteins [considering the number of protein for these extreme proteins are less than neutral proteins ranged from 5-7] as shown below.

In general, the IPG strips are advantageous over the ampholyte method in its ability to avoid cationic accumulation and production of better focused protein with less smearing (Bjellqvist et al, 1982).

3.1.1 Conventional IEF

Proteins are amphoteric molecules; they carry either, positive, negative or zero net charge, depending on their pH of their surroundings. The net charge of protein is the sum of all negative and positive charges of its amino acids chain side and carboxyl- termini. In conventional IEF, a protein will move to the point where its net charge is zero. The original method of IEF depends mainly on carrier ampholyte- generated pH gradient in polyacrylamide gel rods in tubes. When electric current is turned on, the ampholytes with highest PI will move to the anode and vice versa. The main disadvantage of this method is that ampholytes have some tendency to drift towards cathode. This gradient drift usually causes reduction in reproducibility. Together with low mechanical stability, batch-to-batch variability, and the probability of the soft gel to be stretched or broken, Immobiline pH gradient was introduced by Gorg and colleagues (Gorg, 1993; Gorg et al, 1995; Gorg et al, 1998; Gorg et al, 2000; Gorg et al, 1992; Gorg et al, 1988b). More description can be found in section 3.1.3.

3.1.2 Non-equilibrium pH gel electrophoresis (NEPHGE)

This technique has been developed for resolving proteins with extremely high PI (~ 7.0 to 11.0) (Lopez, 1999; O'Farrell, 1975) that cannot be resolved by conventional IEF due to their extreme PI nature. In this technique, and unlike IEF, the proteins move at different rate across the gel owing to the charge and the volt hours setting determine the speed pattern and reproducibility. The procedure of NEPHGE is similar to that in IEF. However, in NEPHGE, the polarity of 1DE is reversed by using an adapter that attaches the power supply. More details can be found in (Lopez, 1999). According to (Lopez, 1999), 4 critical consideration should be taken in mind when separating proteins using NEPHGE:

1. Although the NEPHGE procedure separates proteins with basic PIs, it is best to use a wide-range ampholyte mixture. A basic mixture of ampholytes will crowd the acidic proteins in a narrow region and potentially obscure some of the basic proteins.
2. For the NEPHGE procedure, the positions of the anode and cathode buffers are reversed. This results in a better separation of the basic proteins.
3. During NEPHGE, the IEF gels are not actually "focused" to their pIs. The separation is based on the migration rates of the differentially charged polypeptides as they move across the gel. Therefore, it is necessary to pay strict attention to accumulated volt-hours during the run to assure reproducible patterns in subsequent separations. During the pre-run, a pH gradient is set up, and the focusing voltage is reached. The resistance in the gels is such that this voltage should be achieved in <2 h. If it takes longer, the samples may have high conductivity or the ampholyte quality may be inferior.
4. The conditions for optimum polypeptide separation will most probably have to be empirically determined for the NEPHGE gels. This is because the proteins are not focused to their respective pIs as in standard IEF. Therefore, a series of test runs with different accumulated volt-hours should be compared to optimize for each sample.

3.1.3 Immobilized pH gradient (IPGs)

A ready- made immobiline IPG strips are now available with different length. Usually, short length IPG strips are used for fast screening while longer one for maximal and comprehensive analysis. As mentioned previously, a commercial pre-casted acrylamide gel matrix copolymerized with a pH gradient on a plastic strip results in a stable pH value over the traditional ampholyte method.

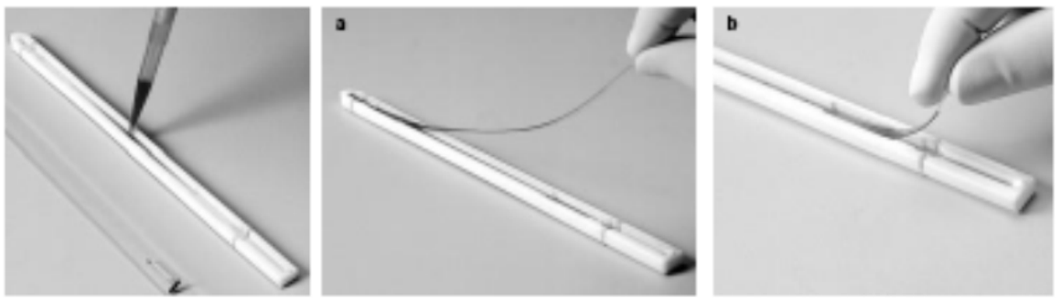


Fig. 4. The isoelectric focusing starts with application of the sample dissolved in rehydration buffer followed by inserting the IPG strip and covering with an oil layer to prevent evaporation. The assembly is then placed in a device that generates an electric gradient for focusing (ex, Ettan IPGphore) [from Amersham manual].

3.2 Second dimension: SDS PAGE

This step separates proteins based on their molecular weight using a vertical electrophoretic device and using either Laemmli buffer (Laemmli, 1970) or Tris- Tricine buffer (Rozalska & Szewczyk, 2008). Instead of loading protein sample within the wells, the 1st dimension rehydrated strip is placed on the top of the SDS-PAGE and sealed with agarose. More information can be found in (Magdeldin et al, 2010).

Gentle	Mechanism	Condition	Suitable samples	Comments
Cell lysis by osmotic shock	Suitable for fractionating subcellular organelles	Water to prewashed cells with a ratio [2:1]	Bacteria	Lower protein yield
Detergent lysis	Souliblize cellular membranes	Suspend in detergent buffer	bacteria	
Homogenization	Using hand homogenizer to physical break down cells	Chop tissue, add chilled buffer * filtrate and centrifuge	Soft tissues (Liver)	Proteases is liberated
Enzymatic digestion	Digest cell wall	Lysozyme at 0.2µg/µl	bacteria	Combined with mechanical disruption

Table 1. Mild (gentle) cell homogenization. * add chilled homogenization buffer in a ratio of [5: 1 v/v].

Moderate & Strong*	Mechanism	Condition	Suitable samples	Comments
Freezing & Thawing	Cells are subjected to cycles of freeze/ thaw	Freeze cells, thaw, repeat several time		May affect the integrity of proteins
Blade homogenization	Mechanical tearing of tissue	Follow device instruction	Most animal tissues	
Grinding with beads	Beads mechanically destruct the cells	1-3 g glass beads, vortex	Bacteria and plant cells, animal tissues	
***Ultrasonication**	Ultrasonic waves disrupt cells	Sonicate in ice several times each 15 sec	All samples	Release of nucleic acids [add DNase]
***French press**	Cells are disrupted by forcing it through a small orifice under high pressure	Place cell suspension in chilled French pressure cell	Bacteria and plant cells	

Table 2. Moderate and strong (vigorous) cell disruption methods.

4. Sample preparation of 2-DE

Sample preparation is a critical step for obtaining a reproducible and informative 2-DE result. The addition of SDS, an anionic detergent, is one of the most effective surfactant to solubilize proteins and inactivating proteolytic enzymes simultaneously. However, it cannot be used in IEF to separate proteins based on their charge as it interfere with isoelectric focusing. Instead, nonionic detergents (NP-40 or Triton X-100) have been considered the best choice for extracting proteins aimed to be processed by 2- DE later. The original lysis buffer for using in 2D-PAGE (O'Farrell, 1975) contains 9.5 M urea, 2% Nonidet P-40, 0.8- 2.0% (w/v) ampholytes of various pH ranges, and a reducing agent such as 2% mercaptoethanol (2-ME) or DTT. For dissolve membrane proteins from tissue samples, various of chaotropes for example thiourea or a combination of Urea and thiourea (7 M and 2 M), respectively (Musante et al, 1998), zwitterionic detergent (Chevallet et al, 1998) showed a better solubilization of these hydrophobic proteins. We provide a simple introduction here based on our experience in 2-DE separation.

4.1 Protein extraction

Several receipts of sample buffer preparation (termed hereby rehydration buffer) are reported based on the presence of different chaotropes, non-ionic detergents and others. In general, the formula of the rehydration buffer can be modified based on the experimental condition and targeted proteins of interest.

Depending on the starting sample, several methods of extraction mechanism may be used which is ranged in its severity from mild (gentle), moderate and strong. The table shown below, summarize the most commonly used methods for cell disruption and cell homogenization

4.2 Removal of non-protein contamination

Before starting first dimension electrophoresis (IEF), non-proteins contaminants, such as nucleic acids, ionic molecules and lipid, should be removed as they may interfere protein separation in first dimension as well as proteins staining in following steps.

In this context, pre-gel organic solvent proteins precipitation may be necessary to remove lipid. Moreover, nuclease treatment to remove DNA and RNA that can affect gels staining as stick line or efficient sonication may be needed. Other methods such as dialysis in order to remove ionic molecules especially for sample desalting usually improve the final resolution and fractionation of the sample.

4.3 Rehydration buffer

Although rehydration buffers can be purchased directly from many vendors. In many cases, they might not be suitable for a designated experiment. In that case, we cannot modify the component of the buffer unless we prepare it from the scratch. Below is an example of a universal rehydration buffer that has proven to extract a wide range of proteins from different tissues, we frequently use in our 2D lab work.

Urea 7 M, Thiourea 2M, NP-40 (Non- idet) 2% (w/v), Pharmalyte [pH 3-10] 0.2%, DTT 100mM, a collection of protease inhibitors [E-64 250mg/µl, PMSF 100 mM of 10ug/µl, TLCK 10 mg/ µl, aprotinin 2.0 mg/µl, chymotrypsin 1mg/µl] or can be replaced by protease inhibitors cocktail, 0.01% Bromophenol blue.

5. Practical overview of 2-DE (tips and tricks)

Below, we describe in concise points the workflow of 2D gel.

First dimension electrophoresis

1. Load the sample dissolved in rehydration buffer in the ceramic tray [we prefer prior incubation at 37^{o}C before work]. The volume of varies depending on the length of the IPG strip used [125 µl for 7 cm, 200 µl for 11 cm, 250 µl for 13 cm, 340 µl for 18 cm, and 450 for 24 cm].
2. Remove the plastic covering that protects the immobilized gel on the strip from its acidic end carefully.
3. Place the strip in the tray with careful attention to minimize air bubbling. The gel is directed down.
4. Cover the IPG strip with cedar oil 1.2 to 1.4 ml above the IPG strip.
5. Wipe the edges and cover the tray with its plastic covering.
6. Place the ceramic tray carefully in the isoelectric focusing machine (ex. IPGphore 3 isoelectric focusing unit).
7. Adjust the protocol as recommended by the manufacture (modified based on the experiment).
8. Prepare the stock running buffer for 2D. For 10x (1L) dissolve 30 g Tris base, 144 g Glycine, 10 g SDS in 1-liter ultrapure water (milliQ) and stir well.
9. Prepare a fresh 10% ammonium persulphate (APS).
10. Cast and assemble the gel unit as described by manufacturing protocol. All equipments should be cleaned, washed with milliQ and pre-dried.

11. Preparing the gel [all reagent should be kept in room temperature at least 30 minutes prior to gel preparation]
12. In a conical flask, prepare the required volume of polyacrylamide gel depending on the number and size of gel to be processed. Below is an example for 24 cm 2D gel.
13. Mix all reagents in the table 3 for 5 minutes
14. Add TEMED (per microliter) according to the number of gel prepared [80 µl, 100, 120, or 140 for 2, 3, 4, or 5 gels], respectively.
15. Add 10 % APS (per milliliter) according to the number of gel prepared [1 ml, 1.2, 1.5, or 1.75 for 2, 3, 4, or 5 gels], respectively.
16. Mix well for 3 minutes and pour the gel slowly into the cast.
17. Remove air bubble by gentle sticking the gel on the ground.
18. Add few milliliter of MilliQ of the top of the gel to prevent gel drying.
19. Protect the gel by plastic covering and allow polymerizing for at least 1 hr.
20. Prepare IAA and DTT for equilibration buffer. Weigh in1.5 ml Eppendorf tube 250 mg and 100 mg of IAA and DTT, respectively. Keep them until use.
21. To prepare the working running buffer (from the 10 x previously prepared in point 8), 1 x running buffer if prepared by diluting 200 ml of the stock (10 x) with 1800 ml MilliQ, pour this diluted buffer in the 2D tank and keep it cold.
22. Prepare 2 X running buffer by diluting 400 ml of the 10 x stock with 1600 ml MilliQ. This 2 different running buffer are placed in the cathode and anode for generating an electric current.

Reagent	Gel number 2	3	4	5
Separation buffer*	50 ml	62.5	75	87.5
30% acrylamide (0.8% BIS)	83.3 ml	104	125	145.5
50% Glycerol	33.3 ml	41.5	50	58.5
10 % SDS	2 ml	2.5	3	3.5
MilliQ	31.4 ml	39.5	47	55

* Separation buffer [1.5 M Tris, pH 8.8, filtered]

Table 3. Preparation of polyacrylamide gel for 2D.

Second dimension electrophoresis

1. After performing the isoelectric focusing, remove the IPG strip carefully, wash it with MilliQ to remove the oil.
2. Perform the reduction of the strip by dissolving DTT (point 20) in 10 ml rehydration buffer (point 5), place the IPG strip in the equilibration buffer, and incubate in a shaker for at least 20 minutes.
3. Perform alkylation step by dissolving IAA in rehydration buffer, and incubate in a shaker and dark place for 20 minutes.
4. For both reduction and alkylation steps, the gel side in the strips should be directed up.
5. For preparing rehydration buffer [6M urea, 50mM Tris- HCl, pH 8.8, 30% Glycerol, 2% SDS, 0.004% BPB]. Aliquot in 10 ml and store at -20 ^{o}C
6. After reduction and alkylation of IPG strips, place the IPG strip on the edge of the prepared gel (require experience and skill).

7. Pre- prepared and melted sealing agarose should be available to seal the gaps between the gel in the IPG strip and prepared gel used for 2nd electrophoresis. To prepare the sealing agarose, 0.5% agarose is required. Mix 10 ml running buffer (10 x) plus 90 ml MilliQ and 0.5 g pure agarose and microwave the mixture and add from 200 to 300 ml BPB. Finally, aliquot in eppendorf tubes.
8. Add 1 ml of the sealing agarose on the IPG strip. Leave to polymerize around 10 minutes.
9. Place the gel in the electrophoresis device.
10. Add the 2 x running buffer on the top of the gel
11. Run the electrophoresis by a rate of 2w/gel. Observe the front dye of the BPB in the sealing agarose to reach to the desired place.

6. Protein visualization and staining

A large number of methods are available for protein detection, which can be used either in SDS-PAGE or in 2-DE. Most of them involve binding of the dye of the stain ions to the protein. With the great variation in detection efficiency, accuracy to quantify protein amount, compatibility with the mass spectrometry, complexity of procedures, and even its cost, the choice of the stain depends mainly on the experimental workflow. In general, there are 4 major categories of protein staining for 2-DE; organic dye staining, classical silver staining and mass spectrometry- compatible silver staining, fluorescent staining, and phosphoprotein stains.

Visualization of 2-DE is somewhat different than SDS-PAGE. Proteins are resolved as a spot instead to discrete band. However, the addition of a second dimension to the gel does not any way hinder visualization, instead it adds a sharpness and defined boundaries to the define spot.

IPG strip	pH	Silver stain	Coomassie stain
7 cm	4-7	4-8	20-120
	6-11	8-16	40-240
	3-10, 3-10 NL	2-4	10-60
11 cm	4-7	10-20	50-300
	6-11	20-40	100-600
	3- 10 L	4-8	20- 120
13 cm	4-7	15- 30	75- 450
	6-11	30- 60	150- 900
	3-10, 3-10 NL	8- 15	40- 240
18 cm	4- 7	30 -60	150- 900
	6- 11, 6- 9	60- 120	300- 1500
	3- 10, 3- 10 NL	15- 30	75- 450
24 cm	4- 7, 3- 7	45- 90	200-1300
	6- 9	80- 170	400- 2000
	3- 10, 3- 10 NL	20- 40	100- 600

Table 4. Suitable sample loads for silver and coomassie staining.

6.1 Organic dye staining

Organic dye refers to coomassie blue R and G types. Chemically, colloidal coomassie blue G-250 stain (CBB) consists of triphenylmethane, which differs from brilliant blue by the addition of two methyl groups. G, variant refers that the blue stains has a slight green tint. While the 250 refers to the purity (Chial et al, 1993). CCB has a limited protein detection ranged from 8- 50 ng within the spot. A major advantage of CBB is the compatibility with mass spectrometry (Neuhoff et al, 1988). The dye complexes with basic amino acids, such as tyrosine, histidine, arginine, and lysine. The formation of protein- dye complexes stabilizes the negatively charged anionic form of the dye producing a clear protein spot with faint background color. That is because the property of colloidal nature of the stain prevents it to penetrate to the gel. On binding to protein, a negative charge of the dye will dominate the protein – dye complexes. This feature can be used to separate proteins and protein complexes under denaturating condition using polyacrylamide gel electrophoresis in a technique called Blue – native gel (Schagger & von Jagow, 1991; Wittig et al, 2006).

6.2 Silver staining

Silver staining was first introduced by (Switzer et al, 1979) . Since that time, it has been widely used and it became more popular. Among the various protein detection methods following 2-DE, silver staining has gained wide popularity because of its sensitivity. Ammoniacal silver stain provides a high sensitivity (2-4 ng) over CBB staining [30 – 100 times more sensitive]. The high sensitivity of silver staining makes it more susceptible to interference with other reagents even the purity of water. We can imagine that usage of distilled water can give an erratic result. Instead, deionized water should be used. The rationale of silver staining is quite simple. Proteins bind silver ions, which can be reduced under appropriate conditions to build up a visible image made of finely divided silver metal. Among silver- ammonia (ammoniacal) or silver- nitrate, the later are simplest in term of experimental procedures and speed. However some disadvantages including that basic protein are less efficiently stained than acidic ones (Chevallet et al, 2006; Lelong et al, 2009). Moreover, They do not work properly below 19–20°C, except when the water used for making the solutions from steps is warmed at 20–25°C or above prior to use.

A major drawback of silver staining that it is not compatible with mass spectrometry. This is because proteins within the gel to be analyzed should remain in its unmodified status. Recently, kits for MS- compatible silver staining are available.

6.3 Fluorescent staining

Highly sensitive fluorescent stains that include different families like (SYBRO, CyDye, and Deep purple). These flurophors have a wide liner range of detection which facilitates a powerful and accurate quantitative evaluation of the sport intensity (Patton, 2002). These dyes are currently available commercially and ready to be used to analyze protein lysate, from bacterial and mammalian cells. Their sensitivities are slightly higher or comparable to silver staining but not as high as radiolabel ling.

Deep purple stain can detect protein spot as 0.5 ng. It is compatible to mass spectrometry. This stain can be used with ultraviolet transillumination (365 nm), light boxes (400-500 nm), laser- based scanners (457, 488, or 532 nm excitation), and CCD cameras.

Sybro Ruby stain is high sensitive stains. It is a ruthenium- based metal chelate fluorescent stain. The ruby stain has been shown to detect 20% more protein compared to silver stain (Lopez et al, 2000). It has a liner dynamic ranges that covers three order magnitudes. Also its merit over silver stain in its short processing time and need no fixation step prior to staining.

CyDye provides a multicolor dye staining. cy2, cy3, and cy5, which exhibit different excitation and emission spectra, can be used in differential gel electrophoresis (DIGE). Providing an excellent quantitative interpretation for protein spot analysis. Its rather expensive and need to be analyzed in an over layed computerized method (Alban et al, 2003).

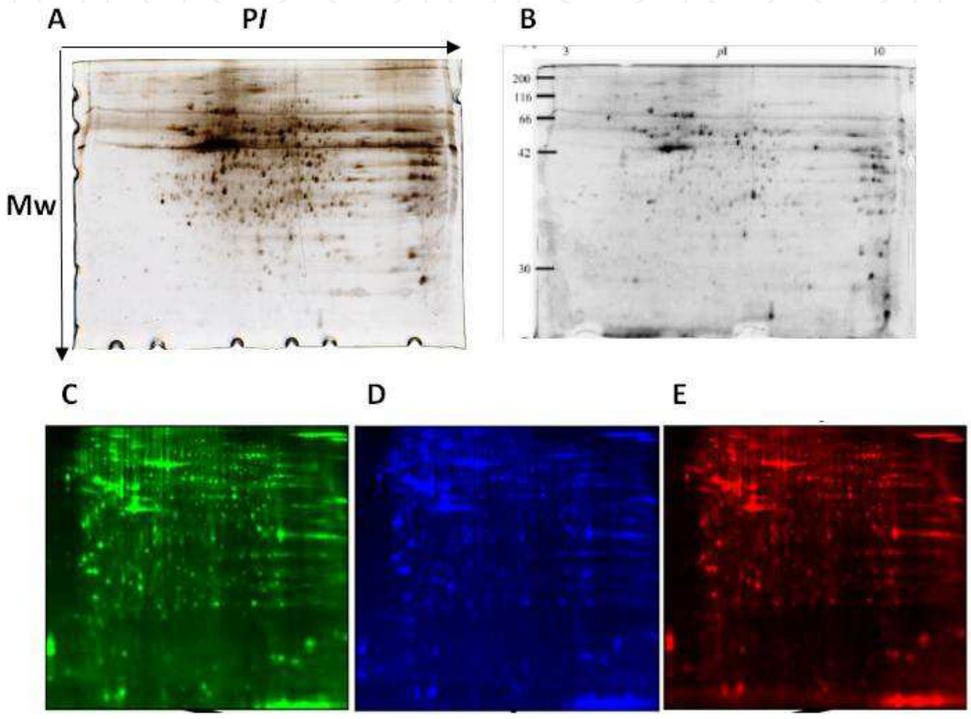


Fig. 5. Staining of 2-D gels. A) 24 cm two dimensional polyacrylamide gel electrophoresis of mouse colon protein stained by silver staining or (B) Deep purple flurophore dye. Visualization of B image was done using a laser scanner (Magdeldin et al, 2010). C, D, and E shows 2D-DIGE stained with cyDye (cy2, cy3 and cy5), respectively.

6.4 Phosphoprotein and glycoprotein stains

A colorimetric stain to specifically detect proteins separated by polyacrylamide gel electrophoresis (SDS-PAGE) or 2-DE. Protein of interest that is phosphorylated at serine and/or threonine residues can be detected using these stains. Staining is achieved by first hydrolyzing the phosphoprotein-phosphoester linkage using 0.5N NaOH in the presence of calcium ions. The gel containing the newly formed insoluble calcium phosphate is then treated with ammonium molybdate in dilute nitric acid. The resulting insoluble nitrophospho-molybdate complex is finally stained with a solution of the basic dye, methyl green. In glycosylated proteins, separated proteins is treated with a periodate solution, which oxidizes cis-diol sugar groups in glycoproteins. The resulting aldehyde groups are

detected through the formation of Schiff-base bonds with a reagent that produces magenta bands (PAS staining). Several commercially available stains for both modifications such as (Pro-Q Diamond, Gelcode, Pro-Q Emerald or AMRESCO) are now available. These types of stains are of great significance for detecting post- transitional modification and understanding disease-related proteins.

7. The state of art in the analysis of 2-DE images

Currently, the bioinformatics usage of a variety of softwares enabled easy and accurate comparison of several 2D gels at once. Assuming the presence of hundreds to thousand(s) of spots within a single 2D gel. Manual comparison became impossible. The state of art for analysis of 2D gels in a comprehensive way using some softwares became the replacement for traditional manual checking.

7.1 Historical advances in the analysis of 2-DE images

The application of powerful and speedy software systems has been enabled by a series of improvements in 2-DE gel image acquisition and process technology over the last decades. The first computer-based analysis systems emerged in the late 1970s was introduced without a graphical user interface. Since the mid-1980s, programs used X-Windows-based graphical user interfaces on computer workstations have been developed. In 1989, Nonlinear (Newcastle, UK) introduced the first 2-DE gel analysis software running on desktop PCs instead of workstations.

While until then none of the available systems could process spot matching for different gel images, Melanie II was the first which introduced image adjustment based on a global polynomial transformation of the image's geometry (Horgan et al, 1992). They developed a strategy for the first time of superimposing of false-colored 2-DE images to simplify the finding of differences in spot patterns. This technology was further improved by establishing positional correction by image warping of the raw 2-DE gel images and commercialized with the first version of the Delta2D software (Greifswald, Germany), coinciding with Compugen's Z3 software in 2000. This simplifies and speeds up analysis dramatically but still produces expression profiles with information gaps resulting in unreliable protein expression analysis.

With the ever-increasing capacity of available computer hardware, more advanced image-processing methods became possible. So far, Progenesis SameSpots (Nonlinear Dynamics) developed a technology to produce a complete set of gel images for an experiment as all gels contain the same number of spots, each matches to the corresponding spot on all gels. In addition, Delta2D (Decodon) has introduced an algorithm to integrate the information of all gel images of an experiment into a so-called fusion gel image, which makes it possible to generate a proteome map that is representative for the whole experiment. Therefore, there are no missing values in the both advanced softwares during the image processing allowing 100% matching spots and valid multivariate statistical analysis to be applied subsequently.

7.2 Workflow of the 2- DE image analysis

The current commercial softwares for 2-DE gel image analysis are listed in Table 5. Besides the commercial available softwares, there are also some freely accessible ones. e.g., FLICKER (http://open2dprot.sourceforge.net/Flicker).

According to the analysis workflow of the software, 2-DE gel, image analysis is usually started with spot detection or image alignment. With the approach beginning from spot detection (e.g. PDQuest and Proteomweaver), the gel image information is first condensed into a set of spots including the information of spot centers, boundaries and volumes for each gel image. Then the spot matching and volume calculation are conducted based on the condense information of each gel image.

Company	Software name	Image analysis approach
Bio-Rad	PDQuest	Spot detection first
	Proteomweaver	Spot detection first
GE Healthcare	Decyder 2D	Spot detection first
	ImageMaster 2D Platinum	Spot detection first
GeneBio	Melanie	Spot detection first
Compugen	Z3 (discontinued)	Image alignment first
Decodon	Delta2D	Image alignment first
Nonlinear Dynamics	Progenesis SameSpots	Image alignment first

Table 5. Current commercial softwares used for 2-DE gel image analysis

This strategy is common way to proceed the analysis considering the computer limitation. For the later approach started with image alignment (e.g. Progenesis SameSpots, Z3 and Delta2D), spot position correction and image warping is first done in order to remove running differences between gels based on the whole gel information. The spot detection, volume calculation, and statistical comparison are then performed after gel image alignment. Performances of the two different image analysis strategies were evaluated and compared by (Millioni et al, 2010) using both standard and experimental gel images. They reported that the true positive spot count and spot matching were higher, while the false negative and positive spot counts were lower when using the approach started with image warping (Delta2D) compared with the one started with spot detection (Proteomweaver). In addition, a smaller amount of analysis time and fewer user interventions were needed with Delta2D compared with Proteomweaver.

The typical workflow of a 2-DE gel image analysis started with the image alignment (e.g., Progenesis SameSpots) can be described as follows (Figure 6) according to (Magdeldin et al, 2010).

1. Perform a biological experiment and prepare appropriate sample for 2-D gel electrophoresis. Note that reproducibility is critical to reduce variations between same group individuals. It is better to run the gel of the same group at once.
2. Separate proteins of a complex sample on a gel according to their pI (isoelectric point) in the first dimension and MW (molecular weight) in the second dimension. A variety of staining techniques can be applied before or after separation to enable spot detection. CBB is not recommended here because of its low dynamic range. Instead, silver or fluorescent staining is recommended.
3. Capture gel images by using scanners or CCD-camera-based image systems. According to different staining dyes and techniques, corresponding imaging devices and

conditions have to be selected for image export making sure of high quality and quantity information.

4. Open 2-DE gel images in the analysis software. In some softwares, image quality check is performed automatically before analysis. For Progenesis SameSpots, at least 16-bit TIFF images are necessary for enough quantitative information.
5. Perform gel image alignment by manual and automatic ways. It is recommended to start with "landmark" spots [spots which are clearly observed and shared within all gels with no confusion]. Usually one image among a series of images of an experiment is chosen as the reference image [the higher number with clearly visible spots] and then the other images are matched to this reference image. As shown in Figure 6D, the spots in the reference image are false-colored in pink while other images in green. The position of green spots could be corrected and aligned to the red ones by clicking the green spots and directly drawing them to the positions of red ones. Usually this process is began with manual operations for several landmark spots and finished with automatic image alignment for other spots in the whole gel. Finally, each gel has a consensus spot pattern for the whole experiment, therefore spot matching reaches 100% without any missing value.
6. After gel image alignment and filtering out certain areas, which are not expected to be included for spot expression analysis, the analyzed images are grouped depending on the experimental design (e.g., Wild and Knockout).
7. Spot detection, volume normalization and quantitation are performed automatically.
8. Build the statistically ranked list of spots according to ANOVA test or fold change and extract proteins of significance in the biological experiment.

7.3 Challenges in the analysis of 2-DE images

Although bioinformatic softwares made 2D gel much easier, several challenges remain in order to achieve a non-biased and reproducible result.

7.3.1 Before software analysis

The fundamental rule in computer-based 2-D gel image analysis is that the quality of the image raw data has a significant impact on the final result. Therefore, it is necessary to produce a reproducible and clearly separated 2-DE gel image in order to avoid getting false positive unreliable results.

Streaks, artifacts, speckles and background would negatively influence the quality of software's work including image alignment, spot detection and quantitation. In addition, if the difference in protein species of two test samples were quite big, the subsequent image alignment between these two images would become very confusing and lack of accuracy. In this case, appropriate control is strongly advisable, for example, preparing a 2-DE gel image of a half-half mixture of two test samples as the reference image for spots' position correction of other gel images. Furthermore, reasons for variation of spot position may be variations on the pH value of the running buffer, problems of incomplete polymerization of the gel, current leakage, or highly abundant proteins that may influence the pH gradient in the IPG gel by their own locally concentrated buffer capacity2. In this case, application of Cyanine dyes to the same gel eliminates the gel-to-gel variance and the internal control can serve as a good reference image (see below).

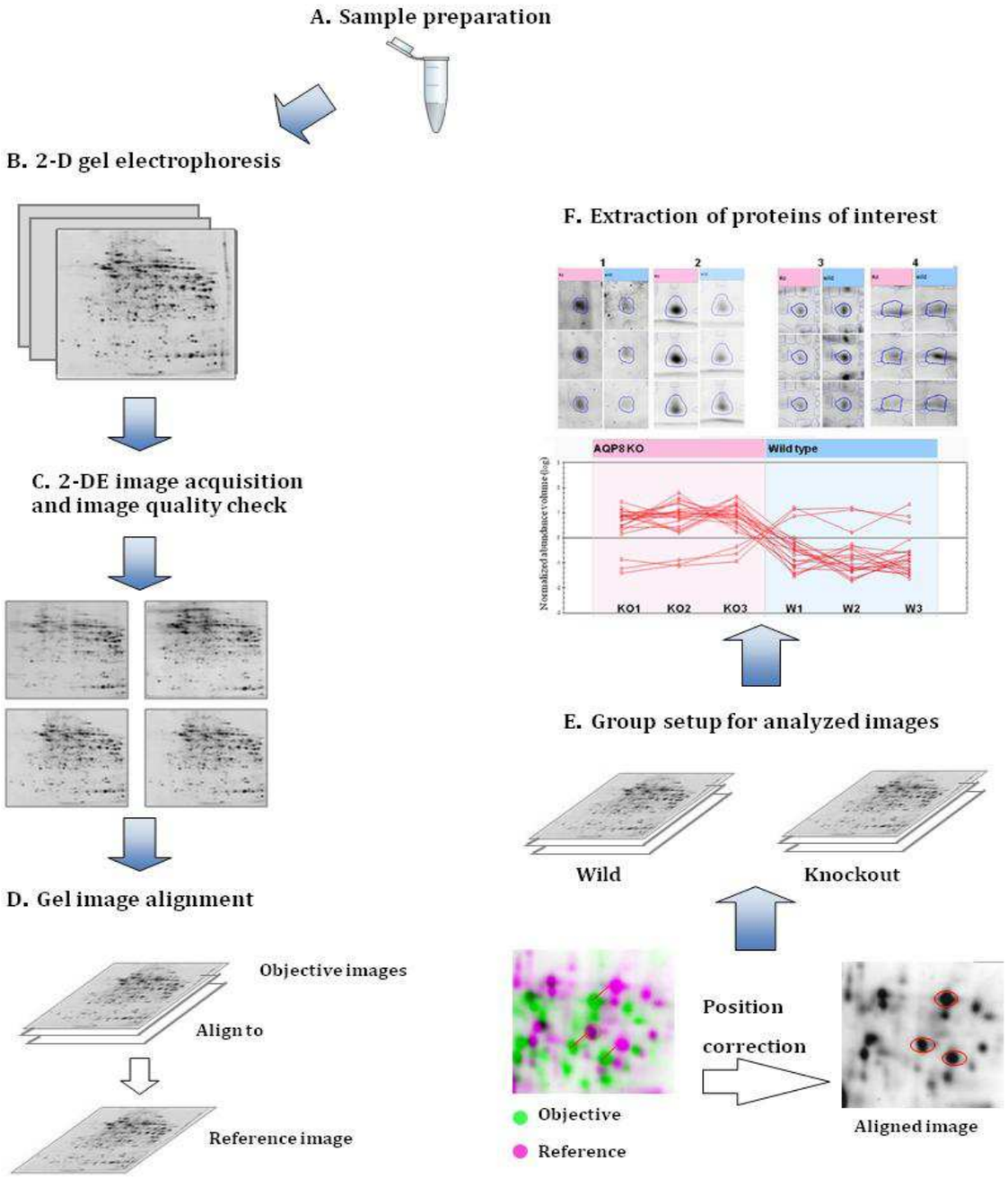


Fig. 6. Image analysis workflow of a 2-D gel electrophoresis based proteomics experiment using Progenesis SameSpots (Nonlinear Dynamics). A. Sample preparation; B. 2-D gel electrophoresis and gel staining; C. 2-DE image acquisition and image quality checked by the software automatically; D. Gel image alignment. A 2-D gel image in the set of images for an experiment is manually set as the reference image (pink) by the user and then other 2-D gel images (green) are aligned to the reference gel image one by one by a manual and/or automated way. E. After gel image alignment, the aligned images are grouped according to the experimental design. F. Extraction of proteins of interest. Spot volume normalization and calculation are performed by the software automatically.

Various dyes are available to make the separated spots detectable. Ideally, a dye for protein stain should bind to the protein with a linear response curve and allow for a detection of very low protein amounts. In fact, the dynamic range of detection depends on the stain used though protein concentrations in biological systems may vary by six or more orders of magnitude. Silver stain has a limited dynamic range with poor stoichiometry, whereas fluorescent labels, such as Sypro Ruby, Ruthenium II tris, Lava Purple, and Deep Purple, have a dynamic range of 10^3 and detection limit 0.1 ng which is suitable for the in-gel protein labeling and for the protein quantification analysis. At the same time, saturation should be avoided because it overlooks co-migrated spots and impedes normalized quantitation. One of the most advanced protein labeling technique is to bind proteins covalently with Cyanine dyes (Cy2, Cy3 and Cy5) before protein separation (see figure 5). There are three merits to do in this way: 1) different samples could be applied to one same gel thus gel-to-gel variation in electrophoresis is removed; 2) the application of internal control speeds up image alignment and increases its reliability greatly; 3) Dye multiplexing allows for a quantitative normalization over several gels by using an internal standard, i.e., a mixture of equal aliquots of every sample in analysis.

The quality of digital 2-DE gel images plays an essential role in the following computerized image analysis, thus the raw image data should be produced in the best possible way. Scanners usually provide higher resolution than CCD cameras while consuming more processing time per image. Many software packages allow for post scan image manipulations. One has to avoid those image manipulations incurring some loss of quantitative information such as some image enhancement operations in some generally used softwares, e.g. Photoshop. It is recommendable to use specialized software (e.g. those packaged with scanner or a 2-DE image analysis program) that understands the characteristics of the file format. For example, it is possible to apply certain filtering algorithm attached to a 2-DE image analysis software to remove the background and noise for correct quantitation and for optimizing the appearance of the image on the computer screen.

7.3.2 In the process of software analysis

Difference in spot positions between gels is a major challenge in image processing because they impede accurate spot matching and thus the construction of expression profiles. The key step for removing this is called gel image alignment in Progenesis SameSpots (or gel image registration in Delta2D) in which certain landmark spots are first aligned to the corresponding ones manually and then other spots are aligned in an automated way. However, in some cases, no complete alignment could be obtained if two patterns are so different. Therefore, at this time one should avoid excessive manual interventions because this would worsen the reliability of image control and reproducibility of the operation between different users. Similarly, in the step of spot detection and spot remodeling (e.g., removing spot, spots merging and spot splitting).

As a result of the quantitation and normalization of spot intensity, one should realize the fact that the relation between original protein quantity in the sample and measured spot intensity is influenced by various intervening factors, including sample loss incurring in the IEF and transfer from IEF to the second dimension, efficiency in protein staining, a protein's staining curve over time, and staining curve over concentration. Given the biochemical diversity of protein molecules, it is to be expected that there are some proteins with a nonlinear relation between concentration and intensity. Therefore, one mainly expects to obtain relatively quantitative results referring to same protein species coming from different samples.

7.4 Concluding remarks

In spite of above mentioned limiting factors, it has been shown that even a 25% change in protein quantity can be reliably detected in most of matched spots by using ruthenium fluorescent dye provided that one can control experimental variation and software-related problems that have unfavorable effects on reproducibility. 2D-DIGE (2-D difference gel electrophoresis) based proteomic techniques have been applied to the study on disease biomarker exploration. Owing to the substantial resolution ability of 2-DE and the dramatic advance in image softwares, 2-DE gel-based proteome analysis is still kept competitive with and complementary to other proteome analysis strategies.

8. Advantages and disadvantages of 2-DE

Despite the development of several parallel technologies, 2-DE is most likely remains one of the best methods for separating proteins within one gel with high resolution and efficiency. As any technique, it holds some advantages and disadvantages. The merits of 2-DE can be concluded in its excellent ability to fractionate proteins into definite protein spots for further identification, defining some post transitional modifications or spliced forms of the same protein. It also allows comparative analysis for up / under regulated proteins. Furthermore, coupling mass spectrometry with 2-DE produce a wealth of information about these analyzed spots.

2-DE is well-suited technique for discovery phase research. With the advantage of 2-DE in separating charge and size isomers of proteins, fractionated proteins can be quantified later on and compare visually or by software.

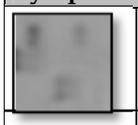
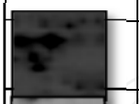
One of the major limitation of 2-DE its limited capability to resolve membrane proteins. Because of the fact that membrane proteins are hydrophobic proteins, it is hardly dissolve in the rehydration buffer containing detergent or chaotropes (Chevalier, 2010). However, using cationic detergent such as benzyldimethyl-n- hexadecylammonium chloride may successfully revolve these membrane proteins in many protein lysate samples (Moebius et al, 2005; Zahedi et al, 2005). Another drawback of 2-DE is the fact that low- abundance proteins are rarely seen because large quantities of proteins available in the sample usually mask its visualization (Greenough et al, 2004; Yamada et al, 2002).

Basic proteins are difficult to be resolved in 2-DE. Strong alkaline proteins near to pI (10.0) such as nuclear proteins can be focused using a wide range strips.

2-DE is more likely to cause some loss in the protein loads. It is a general concept of electrophoresis. High molecular weight proteins are very difficult to be visualized on 2-DE. It was demonstrated that capabilities of 2-DE is good in separating proteins with molecular mass as large as 500 KDa (Koga, 2008).

A frequent criticism of 2-DE is that it is s time consuming and require an expertise researcher especially if you are working with large sized gel (24 cm). Although automated equipments are now available, it remains expensive and requires additional improvements.

In conclusion, despite some drawbacks of 2-DE, the uniqueness of this methodology to visualize protein spots with annotation of its quantity and chemical nature when coupling with mass spectrometry renders this technique one of the most powerful informative approach for direct targeting of protein expression differences.

Symptom (observation)		Possible cause	Remedy§
	**No visible spots**	1. sample amount is low 2. poor sample solubilization	1. increase protein load 2. modify rehydration buffer
	Individual protein is unclear, wrong positioned	1. Protein carbamylation* 2. Protein oxidation	1. do not heat urea solution above 30 °C 2. ensure addition of DTT and IAA
	**Vertically doubled spots**	1. IPG strip is not placed correctly*	1. ensure correct placement of IPG strip when performing 2-DE
	**Distorted pattern**	Unflat surface of the gel	1. overlay milliQ layer over the gel after pouring
	**Horizontal streaking**	1.Incomplete solubilization* 2. Impurities interfering with IPG focusing* 3. High sample load 4. ionic detergent in sample 5. Incomplete focusing Under focusing *	1. increase detergent, solubilization component 2. precipitate and re-solubilize or use cleaning kit 3. reduce sample load 4. reduce salt concentration or limit the voltage of IEF to 150 V/ 2 hrs then resume normally 5. Prolong focusing time
	**Vertical streaking**	1. insufficient equilibration 2. in sufficient SDS in the buffer	1. prolong equilibration time 2. Ensure correct and fresh preparation with addition of 0.1% W/V SDS
	**Vertical gap in the gel**	1. Impurities in sample rehydration buffer 2. bubbles between gel surface and IPG strip * 3. urea crystals	1. modify the rehydration buffer 2. ensure close contact between the strip and the gel 3. allow residual equilibration solution before placing IPG strip
	Point streaking	Silver stain impurities or unclean materials	Ensure proper cleaning of all equipments
	**High background coloring**	Protein contaminant in SDS	Prepare fresh SDS
	**Vertical poor focusing**	IPG strip is not completely hydrated	Hydrate completely and allow enough time, remove bubbles and ensure solution pass through the strip all the time
	**Horizontal incomplete focusing**	Ionic impurities in sample	The final concentration of SDS should not exceed 0.25% after dilution. The concentration of non ionic detergent should be at least 8 times higher than ionic detergent

* Most common trouble shooting

Table 6. Summarized table from Amersham 2-DE manual trouble shooting with modification.

9. Two dimensional electrophoresis trouble shooting

Several problems may be encountered during 2-DE experimentation. As mentioned earlier, 2-DE requires skilled and trained persons. Trouble shooting can be greatly reduced if the researcher followed instruction manuals with careful ensuring that all equipments are clean. Below are most common problems that arise during working with its possible reason and remedy.

10. Concluding remarks

Protein separation is a core part of proteomics analysis and 2-DE is a basic and fundamental procedure to separate each protein from protein complexes. The 2-DE method is superior to show each protein as a spot visually, which make the results or understandings based on the procedure more confident. Although the procedure was empirical and the sensitivity was not so high, the recent advances have mostly overcome these problems. This chapter provides the principle and practical procedure of the 2-DE for the readers. We hope it may help beginners, who want to separate proteins by 2-DE, and for researchers, who have difficulties in 2-DE, to provide hints on how to solve their problems.

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12. References

Alban A, David SO, Bjorkesten L, Andersson C, Sloge E, Lewis S, Currie I (2003) A novel experimental design for comparative two-dimensional gel analysis: two-dimensional difference gel electrophoresis incorporating a pooled internal standard. Proteomics 3: 36-44

Anderson NG, Matheson A, Anderson NL (2001) Back to the future: the human protein index (HPI) and the agenda for post-proteomic biology. Proteomics 1: 3-12

Bjellqvist B, Ek K, Righetti PG, Gianazza E, Gorg A, Westermeier R, Postel W (1982) Isoelectric focusing in immobilized pH gradients: principle, methodology and some applications. J Biochem Biophys Methods 6: 317-339

Bossi ML, Bossi O, Gelfi C, Righetti PG (1988) Casting immobilized pH gradients into cylindrical polyacrylamide gels. J Biochem Biophys Methods 16: 171-183

Castillejo MA, Fernandez-Aparicio M, Rubiales D (2011) Proteomic analysis by two-dimensional differential in gel electrophoresis (2D DIGE) of the early response of Pisum sativum to Orobanche crenata. J Exp Bot

Chevalier F (2010) Highlights on the capacities of "Gel-based" proteomics. Proteome Sci 8: 23

Chevallet M, Luche S, Diemer H, Strub JM, Van Dorsselaer A, Rabilloud T (2008) Sweet silver: a formaldehyde-free silver staining using aldoses as developing agents, with enhanced compatibility with mass spectrometry. Proteomics 8: 4853-4861

Chevallet M, Luche S, Rabilloud T (2006) Silver staining of proteins in polyacrylamide gels. Nat Protoc 1: 1852-1858

Chevallet M, Santoni V, Poinas A, Rouquie D, Fuchs A, Kieffer S, Rossignol M, Lunardi J, Garin J, Rabilloud T (1998) New zwitterionic detergents improve the analysis of membrane proteins by two-dimensional electrophoresis. Electrophoresis 19: 1901-1909

Chial HJ, Thompson HB, Splittgerber AG (1993) A spectral study of the charge forms of Coomassie blue G. Anal Biochem 209: 258-266

Corthals GL, Wasinger VC, Hochstrasser DF, Sanchez JC (2000) The dynamic range of protein expression: a challenge for proteomic research. Electrophoresis 21: 1104-1115

Gianazza E (1999) Casting immobilized pH gradients (IPGs). Methods Mol Biol 112: 175-188

Gorg A (1993) Two-dimensional electrophoresis with immobilized pH gradients: current state. Biochem Soc Trans 21: 130-132

Gorg A, Boguth G, Obermaier C, Posch A, Weiss W (1995) Two-dimensional polyacrylamide gel electrophoresis with immobilized pH gradients in the first dimension (IPG-Dalt): the state of the art and the controversy of vertical versus horizontal systems. Electrophoresis 16: 1079-1086

Gorg A, Boguth G, Obermaier C, Weiss W (1998) Two-dimensional electrophoresis of proteins in an immobilized pH 4-12 gradient. Electrophoresis 19: 1516-1519

Gorg A, Obermaier C, Boguth G, Harder A, Scheibe B, Wildgruber R, Weiss W (2000) The current state of two-dimensional electrophoresis with immobilized pH gradients. Electrophoresis 21: 1037-1053

Gorg A, Obermaier C, Boguth G, Weiss W (1999) Recent developments in two-dimensional gel electrophoresis with immobilized pH gradients: wide pH gradients up to pH 12, longer separation distances and simplified procedures. Electrophoresis 20: 712-717

Gorg A, Postel W, Baumer M, Weiss W (1992) Two-dimensional polyacrylamide gel electrophoresis, with immobilized pH gradients in the first dimension, of barley seed proteins: discrimination of cultivars with different malting grades. Electrophoresis 13: 192-203

Gorg A, Postel W, Domscheit A, Gunther S (1988a) Two-dimensional electrophoresis with immobilized pH gradients of leaf proteins from barley (Hordeum vulgare): method, reproducibility and genetic aspects. Electrophoresis 9: 681-692

Gorg A, Postel W, Gunther S (1988b) The current state of two-dimensional electrophoresis with immobilized pH gradients. Electrophoresis 9: 531-546

Gorg A, Postel W, Gunther S, Friedrich C (1988c) Horizontal two-dimensional electrophoresis with immobilized pH gradients using PhastSystem. Electrophoresis 9: 57-59

Gorg A, Weser J, Westermeier R, Postel W, Weidinger S, Patutschnick W, Cleve H (1983) Isoelectric focusing with immobilized pH gradients for the analysis of transferrin (Tf) subtypes and variants. Hum Genet 64: 222-226

Greenough C, Jenkins RE, Kitteringham NR, Pirmohamed M, Park BK, Pennington SR (2004) A method for the rapid depletion of albumin and immunoglobulin from human plasma. Proteomics 4: 3107-3111

Heywood WE, Madgett TE, Wang D, Wallington A, Hogg J, Mills K, Avent ND (2011) 2D DIGE analysis of maternal plasma for potential biomarkers of Down Syndrome. Proteome Sci 9: 56

Horgan G, Creasey A, Fenton B (1992) Superimposing two-dimensional gels to study genetic variation in malaria parasites. Electrophoresis 13: 871-875

Kaltschmidt E, Wittmann HG (1970) Ribosomal proteins. VII. Two-dimensional polyacrylamide gel electrophoresis for fingerprinting of ribosomal proteins. Anal Biochem 36: 401-412.

Klose J (1975) Protein mapping by combined isoelectric focusing and electrophoresis of mouse tissues. A novel approach to testing for induced point mutations in mammals. Humangenetik 26: 231-243

Koga K (2008) G-electrode-loading method for isoelectric focusing, enabling separation of low-abundance and high-molecular-mass proteins. Anal Biochem 382: 23-28

Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227: 680-685

Lelong C, Chevallet M, Luche S, Rabilloud T (2009) Silver staining of proteins in 2DE gels. Methods Mol Biol 519: 339-350

Lopez MF (1999) Nonequilibrium pH gel electrophoresis (NEPHGE). Methods Mol Biol 112: 129-131

Lopez MF, Berggren K, Chernokalskaya E, Lazarev A, Robinson M, Patton WF (2000) 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 21: 3673-3683

Macko V, Stegemann H (1969) Mapping of potato proteins by combined electrofocusing and electrophoresis identification of varieties. Hoppe Seylers Z Physiol Chem 350: 917-919

Magdeldin S, Li H, Yoshida Y, Enany S, Zhang Y, Xu B, Fujinaka H, Yaoita E, Yamamoto T (2010) Comparison of two dimensional electrophoresis mouse colon proteomes before and after knocking out Aquaporin 8. J Proteomics 73: 2031-2040

Margolis J, Kenrick KG (1969) 2-dimensional resolution of plasma proteins by combination of polyacrylamide disc and gradient gel electrophoresis. Nature 221: 1056-1057

Mets LJ, Bogorad L (1974) Two-dimensional polyacrylamide gel electrophoresis: an improved method for ribosomal proteins. Anal Biochem 57: 200-210

Millioni R, Miuzzo M, Sbrignadello S, Murphy E, Puricelli L, Tura A, Bertacco E, Rattazzi M, Iori E, Tessari P (2010) Delta2D and Proteomweaver: Performance evaluation of two different approaches for 2-DE analysis. Electrophoresis 31: 1311-1317

Moebius J, Zahedi RP, Lewandrowski U, Berger C, Walter U, Sickmann A (2005) The human platelet membrane proteome reveals several new potential membrane proteins. Mol Cell Proteomics 4: 1754-1761

Musante L, Candiano G, Ghiggeri GM (1998) Resolution of fibronectin and other uncharacterized proteins by two-dimensional polyacrylamide electrophoresis with thiourea. J Chromatogr B Biomed Sci Appl 705: 351-356

Neuhoff V, Arold N, Taube D, Ehrhardt W (1988) Improved staining of proteins in polyacrylamide gels including isoelectric focusing gels with clear background at nanogram sensitivity using Coomassie Brilliant Blue G-250 and R-250. Electrophoresis 9: 255-262

O'Farrell PH (1975) High resolution two-dimensional electrophoresis of proteins. J Biol Chem 250: 4007-4021

Orrick LR, Olson MO, Busch H (1973) Comparison of nucleolar proteins of normal rat liver and Novikoff hepatoma ascites cells by two-dimensional polyacrylamide gel electrophoresis. Proc Natl Acad Sci U S A 70: 1316-1320

Patton WF (2002) Detection technologies in proteome analysis. J Chromatogr B Analyt Technol Biomed Life Sci 771: 3-31

Petrak J, Ivanek R, Toman O, Cmejla R, Cmejlova J, Vyoral D, Zivny J, Vulpe CD (2008) Deja vu in proteomics. A hit parade of repeatedly identified differentially expressed proteins. Proteomics 8: 1744-1749

Rabilloud T, Adessi C, Giraudel A, Lunardi J (1997) Improvement of the solubilization of proteins in two-dimensional electrophoresis with immobilized pH gradients. Electrophoresis 18: 307-316

Rabilloud T, Chevallet M, Luche S, Lelong C (2010) Two-dimensional gel electrophoresis in proteomics: Past, present and future. J Proteomics 73: 2064-2077

Rozalska M, Szewczyk EM (2008) Staphylococcus cohnii hemolysins - isolation, purification and properties. Folia Microbiol (Praha) 53: 521-526

Schagger H, von Jagow G (1991) Blue native electrophoresis for isolation of membrane protein complexes in enzymatically active form. Anal Biochem 199: 223-231

Shevchenko A, Wilm M, Vorm O, Mann M (1996) Mass spectrometric sequencing of proteins silver-stained polyacrylamide gels. Anal Chem 68: 850-858

Switzer RC, 3rd, Merril CR, Shifrin S (1979) A highly sensitive silver stain for detecting proteins and peptides in polyacrylamide gels. Anal Biochem 98: 231-237

Wang DZ, Dong HP, Li C, Xie ZX, Lin L, Hong HS (2011) Identification and Characterization of Cell Wall Proteins of a Toxic Dinoflagellate Alexandrium catenella Using 2-D DIGE and MALDI TOF-TOF Mass Spectrometry. Evid Based Complement Alternat Med 2011: 984080

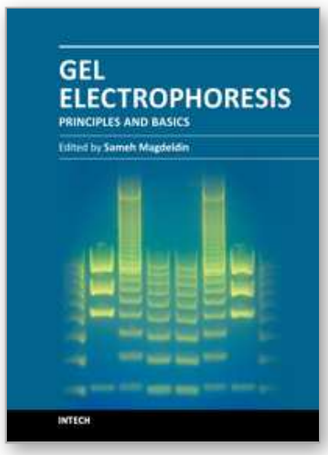
Wilkins MR, Gasteiger E, Sanchez JC, Bairoch A, Hochstrasser DF (1998) Two-dimensional gel electrophoresis for proteome projects: the effects of protein hydrophobicity and copy number. Electrophoresis 19: 1501-1505

Wittig I, Braun HP, Schagger H (2006) Blue native PAGE. Nat Protoc 1: 418-428

Yamada M, Murakami K, Wallingford JC, Yuki Y (2002) Identification of low-abundance proteins of bovine colostral and mature milk using two-dimensional electrophoresis followed by microsequencing and mass spectrometry. Electrophoresis 23: 1153-1160

Zahedi RP, Meisinger C, Sickmann A (2005) Two-dimensional benzyldimethyl-n-hexadecylammonium chloride/SDS-PAGE for membrane proteomics. Proteomics 5: 3581-3588

Zhang Y, Yoshida Y, Nameta M, Xu B, Taguchi I, Ikeda T, Fujinaka H, Mohamed SM, Tsukaguchi H, Harita Y, Yaoita E, Yamamoto T (2010) Glomerular proteins related to slit diaphragm and matrix adhesion in the foot processes are highly tyrosine phosphorylated in the normal rat kidney. Nephrol Dial Transplant 25: 1785-1795



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Most will agree that gel electrophoresis is one of the basic pillars of molecular biology. This coined terminology covers a myriad of gel-based separation approaches that rely mainly on fractionating biomolecules under electrophoretic current based mainly on the molecular weight. In this book, the authors try to present simplified fundamentals of gel-based separation together with exemplarily applications of this versatile technique. We try to keep the contents of the book crisp and comprehensive, and hope that it will receive overwhelming interest and deliver benefits and valuable information to the readers.

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InTech Europe
University Campus STeP Ri
Slavka Krautzeka 83/A
51000 Rijeka, Croatia
Phone: +385 (51) 770 447
Fax: +385 (51) 686 166
www.intechopen.com

InTech China
Unit 405, Office Block, Hotel Equatorial Shanghai
No.65, Yan An Road (West), Shanghai, 200040, China
中国上海市延安西路65号上海国际贵都大饭店办公楼405单元
Phone: +86-21-62489820
Fax: +86-21-62489821

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