

A Simpler and Faster Version of Two-Dimensional Gel Electrophoresis Using Vertical, Mini SDS-PAGE Apparatus

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ABSTRACT: We have modified one of the most useful methods of protein separation; namely, two dimensional gel electrophoresis (2-DE). This modified version of 2-DE is not only simpler and easier but also faster than all the currently available methods. In this method, isoelectric focusing is carried out in the first dimension using a vertical sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) apparatus. Following focusing, each individual lane is excised from this gel and after a 90° rotation, is inserted into a vertical specially fabricated SDS-PAGE gel for the second dimension run, which can be stained with Coomassie Brilliant Blue for protein analysis or immunoblotted for further analysis. This modified version of IEF can be run in less than 2 hours compared to the overnight run required by the O'Farrel method. Difficult tube gel casting and gel extrusion as well as tube gel distortion are eliminated in our method. This method is simpler, faster and inexpensive. Both dimensions can be done on the same SDS-PAGE apparatus and up to 10 samples can be run simultaneously using one gel. We have analyzed human sera, animal saliva, eye and liver tissue samples to verify this method.

KEY WORDS: 2D gel electrophoresis, Isoelectric focusing, Sodium dodecyl sulfate polyacrylamide gel electrophoresis..

INTRODUCTION

Two dimensional gel electrophoresis (2-DE) is a powerful tool for separating proteins based on size and charge. This method is very useful to separate two proteins with identical molecular weights but different charges, which cannot be achieved with just sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). O'Farrel in 1975 introduced the tube-based

isoelectricfocusing (IEF) [1]. In this method, the IEF gel is cast within a glass tube and the protein sample is electrophoresed through it. The pH gradient in the tube gel is made with the use of ampholytes. Following focusing, the tube gel is overlaid on a SDS-PAGE gel and electrophoresed in the second dimension. This method is disadvantageous in that (a) it is relatively difficult to cast

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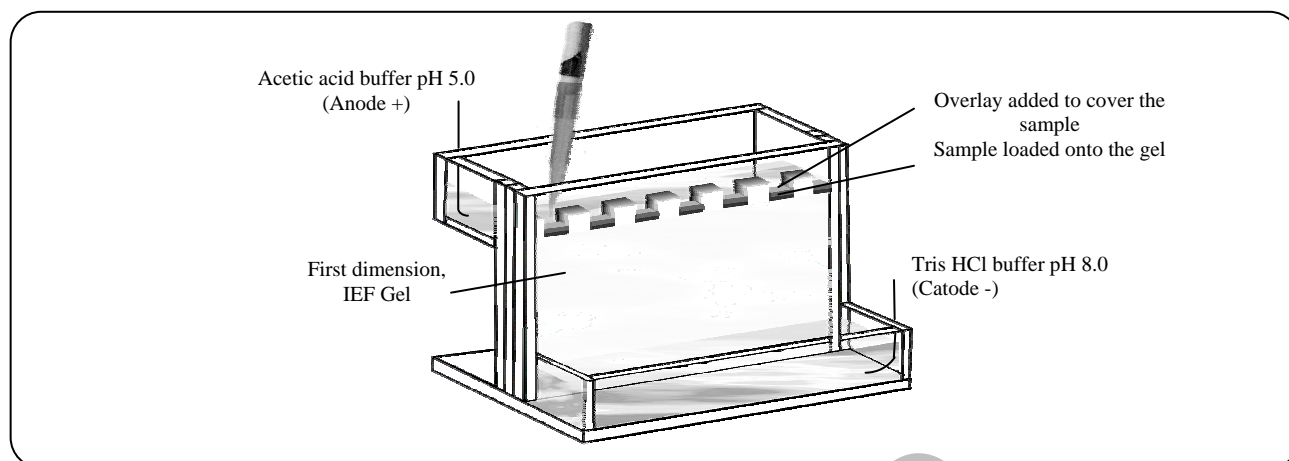


Fig. 1: Schematic representation of first dimension IEF gel. The IEF gel is polymerized like a SDS-PAGE gel and analyzed using Mini Protean II apparatus. A thin film of Spinkote from Beckman was coated along the sides of the apparatus to provide a tight seal and thus prevent mixing of buffers between upper and lower chambers.

the gel (b) the tube gel is difficult to extrude following an IEF run (c) there is the possibility of gel breakage during gel extrusion (d) the gel sometimes slides out of the glass tube during an overnight run and (e) the sample run takes 16 hours.

Other methods have been used to carry out IEF. In flatbed IEF, a non-denaturing gel focusing is carried out horizontally and is not applied to the second dimension. In this method, there is the possibility of sample leakage in between the comb and the gel [2]. Immobilized pH gradient (IPG) phore Isoelectric Focusing System takes 10 hours for the rehydration of IPG strips plus 4 hours of high voltage focusing followed by an overnight run [3]. In one of the newer methods of IEF, the first dimension gel is prepared inside a microporous, non-wetting hollow fiber, and proteins are transported through the wall on to the second dimension. Despite the advantages of this method in eliminating the possibility of distortion of the fragile tube gel from the first dimension to the second, this method still requires force to extrude the gel from the tubes. In addition a high voltage of 2000 V is a necessary factor in this method [4]. Our method also suggests vast improvements over a previously reported vertical isoelectric focusing method [5].

Here we report a modified method that eliminates high voltage focusing, long running time and distortion of the IEF gel while transferring to the second dimension. Our method involves running the IEF gel vertically using SDS-PAGE apparatus. Each individual lane is further excised and electrophoresed in the second dimension

using a SDS-PAGE gel, specially made to accommodate the excised lane. This procedure is faster, simpler and much easier to perform compared to the tube gel or other currently available methods.

MATERIALS AND METHODS

Chemicals

Acrylamide and bis-acrylamide were purchased from Fisher Scientific, NJ. N,N,N',N'-tetramethylethylenediamine (TEMED) and riboflavin were purchased from Sigma Chemical Co., MO. BioLyte 5-8 was from Amersham Biosciences Co., NJ. Ammonium persulfate, urea, Triton x-100, acetic acid, Tris and other chemicals used in this study were of analytical grade.

First dimensional IEF gel

In this method, the IEF gel is cast just like a SDS-PAGE gel that can be electrophoresed using a Mini Protean II gel apparatus (used for mini SDS-PAGE gels) (Fig. 1) [6]. A 4 % IEF gel was prepared [1]. Briefly, the IEF gel was made using 30 % acrylamide/1.5 % bis, 0.004 % riboflavin/ 0.45 % TEMED, 40 % Bio-Lyte 5/8, 1.5 % ammonium persulfate (APS), urea and 20 % Triton x-100. After setting up the apparatus as shown in Fig. 1, the above solutions were mixed and degassed under vacuum for 10 minutes. Gel mix was polymerized by pouring between the glass and backbone plate (using 1.5 mm spacer). A 10-well comb was placed at the top (Fig. 1) and the gel was then placed in front of a UV light overnight at room temperature for polymerization [1].

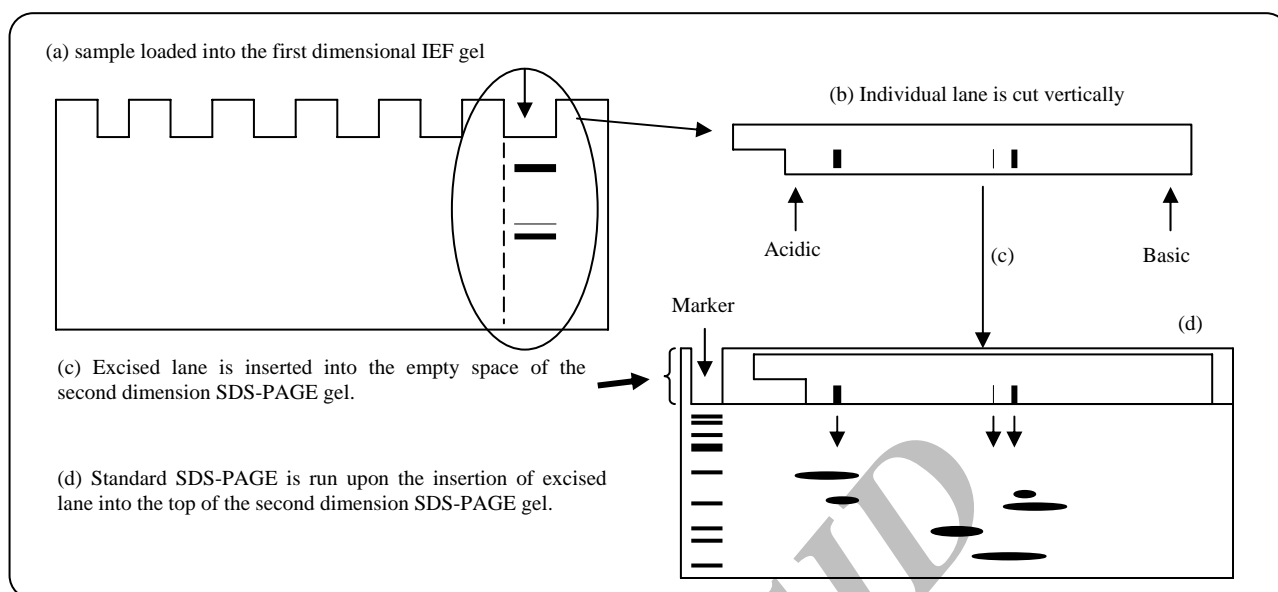


Fig. 2: Schematic representation of 2-DE carried out as mentioned in "Materials and methods":

(a) sample loaded into the first dimensional IEF gel (b) Excision of gel

(c) excised lane is inserted into the empty space of the second dimension SDS-PAGE gel

(d) standard SDS-PAGE is run upon the insertion of excised lane into the top of the second dimension SDS-PAGE gel.

(polymerization can be completed under UV light in 2 hours and we chose the overnight polymerization as a matter of convenience).

Running the first dimension IEF electrophoresis gel

Acetic acid (0.005M, pH 5.0) was used as the acidic buffer (top chamber) and Tris-HCl (0.1 M Tris, pH 8.0) as the basic buffer (lower chamber). The samples were first mixed with lysis buffer (2 M thiourea, 5 M urea, 0.25 % CHAPS, 0.25 % Tween 20, 0.25 % SB 3-10 (Sulfobetaine-10), 0.25 % ampholyte (isoelectric point 5-8), 10 % 2-propanol, 1 mM sodium vanadate, 12.5 % water-saturated isobutanol, 5 % glycerol and β -mercaptoethanol) [7]. The samples were overlaid with overlay buffer (3.0 g of urea, 2 mL of 10 % triton X-100, 0.5 mL of 40 % Bio-Lyte pH 5-8 and made to 10 mL with distilled water) [1].

Sample preparation for the first dimension IEF gel

10 % mouse liver and eye homogenates were prepared as described earlier [8]. The 10 % liver homogenate was subjected to sub-cellular fractionation to obtain the post-nuclear fraction (spun at 800 g to remove the nuclear fraction). Human sera and mouse saliva stored at -20°C were used in this study as well. To run the gel, 25 μL of desired sample is mixed with 75 μL of lysis buffer along

with 25 mg of urea. First, the gel was pre-focused at 100 V (constant voltage) for 50 min. Then 30 μL (Fig. 3A) of each sample were loaded into separate wells. Overlay buffer was added very slowly over the samples, until all the wells were filled to the top, to prevent samples from mixing with the acidic buffer. The upper chamber was filled with acetic acid (pH 5.0) and the lower chamber was filled with Tris buffer (pH 8.0) (Fig. 1). The isoelectric focusing settings were carried out according to *Knisley and Rodkey* [2] with slight modifications. The run was initiated with 100 V (constant voltage) for 15 min followed by 200 V (constant voltage) for an additional 10 min. The settings were then changed to 20 W (constant power) for 30 minutes. Once the last step (the 30-minute step) was over, we observed a yellow horizontal line across the gel right below the wells. The gel was stopped at this juncture.

Preparation for the second dimension

The IEF gel was incubated with equilibration buffer (125 mL of 0.5 M Tris HCl pH 6.8, 20 g of SDS and 50 mL of β -Mercaptoethanol, made up to 1 liter) for 20 minutes. Meanwhile, SDS-PAGE gel (10 % or 12 %) is made with space (1-1.5 cm) at the top for inserting the lane to be excised from the first dimension gel (Fig. 2).

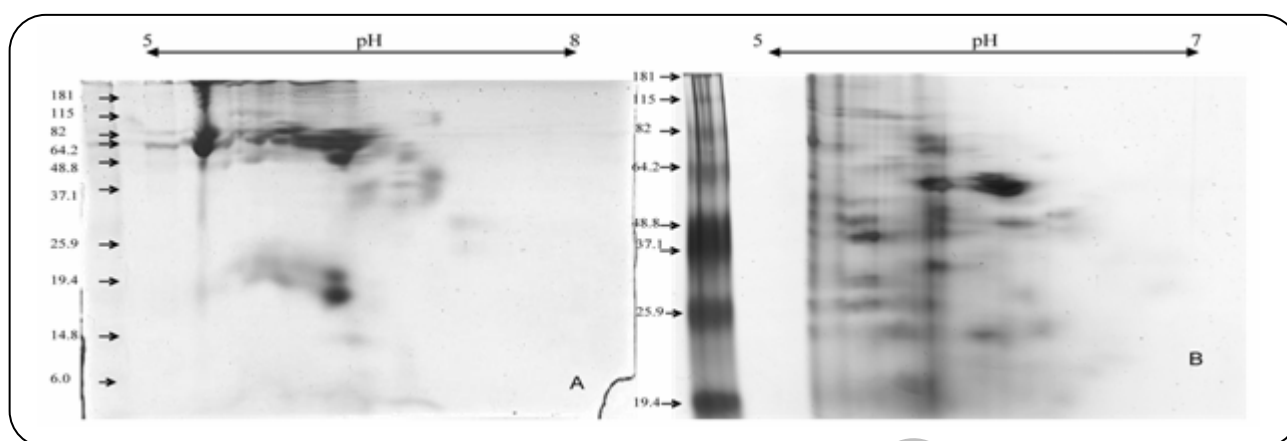


Fig. 3: CBB stained 2-DE gel derived from human sera, and silver stained image of mice liver proteins. (A) human sera (B) mice liver post-nuclear fraction derived from 10 % homogenate.

Second dimensional SDS-PAGE

An individual lane was excised and following a 90° counter clockwise rotation, it was delicately inserted into the top of the SDS-PAGE gel, right inside the empty space. By doing this, the left side of the gel strip represents the acidic side while the right side, the basic end. The same ingredients used for casting the SDS PAGE gel were prepared to pour over the gel strip and fill the space between gel and glass plate. A 2 mm spacer was used for the second dimension SDS-PAGE while the gel lane excised from IEF gel was 1.5 mm thick. This was done to make the insertion of excised gel easy. Once the top portion of the gel is polymerized, the gel is ready to be run. (Meanwhile a well for loading the molecular weight marker was made by inserting a 2 cm long plastic piece (2 mm thick) prior to polymerization of the top gel portion). Once the gel polymerized, standard SDS-PAGE was carried out [6]. The gel could be stained or immunoblotted for further analysis. We stained the gel using 0.05 % Coomassie Brilliant Blue (CBB) overnight, and destained it for 4 hours (Fig. 3A) [9]. Protein spots revealed by silver stain is shown in Fig. 3B [9].

RESULTS AND DISCUSSION

We found that tube gel IEF was very cumbersome and time consuming; therefore we devised a simple method to carry out 2-DE. We have used an SDS-PAGE apparatus (Mini Protean II) to carry out both the first and second dimension gel electrophoresis. We analyzed human sera, mouse liver, mouse eye homogenates and mouse saliva

(data not shown) using this method. Fig. 3A shows a CBB stained 2-DE of human serum sample, analyzed as mentioned in "Materials and methods". After completing the first dimension, the gel was immediately incubated with equilibration buffer for 20 min and then transferred to the SDS-PAGE gel for the second dimension run. Gels analyzed similarly were transferred non-electrophoretically [10] and proteins remaining on the gel were visualized by CBB staining and excised for matrix assisted laser deionization time of flight mass spectrometry [11]. Thus, this method was successfully used in identifying specific proteins (data not shown). Fig. 3B shows a silver stained 2-DE of mouse liver (10 % homogenate). Clear separation of spots can be seen in all Figures. The second dimension for the gel 3B was carried out the day following IEF (the gel remained in-between the two glass plates, covered with a wet tissue paper and wrapped thoroughly with clear plastic wrap to prevent gel from drying, and it was stored at 4 °C overnight. The next day, the second dimension was carried out as mentioned in "Materials and methods"). Horizontal streaking is usually seen in most 2-DE methods [12] due to a variety of reasons such as accumulation of proteins at certain spots (for example, albumin in human serum). We tried to avoid some of this streaking by reducing the width of the excised gel in the first dimension to 3 mm width (Fig. 3B). We have used only mini 10 % gels for the purpose of our experiments however using a gradient and larger gel is definitely recommended for a better resolution in clinical studies.

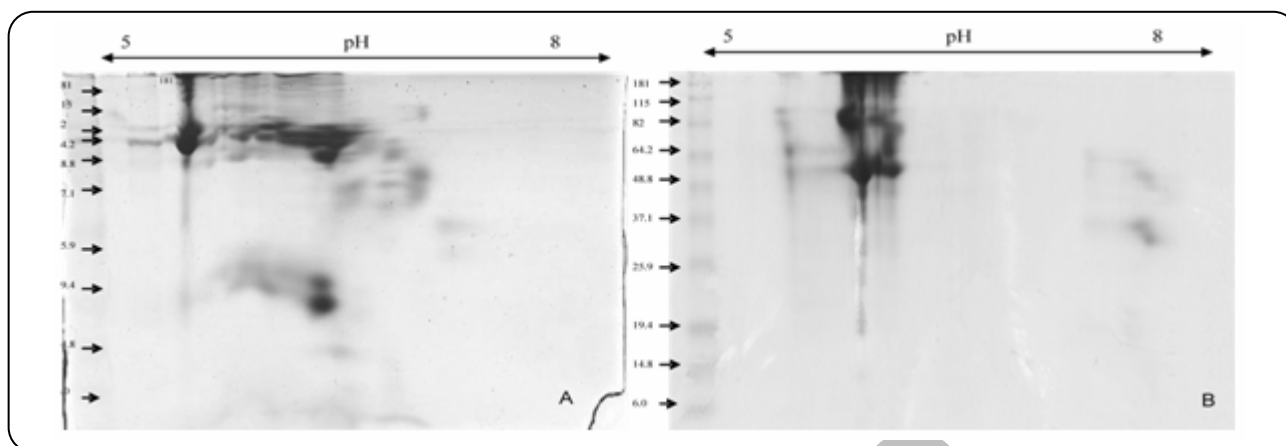


Fig. 4: Comparison between CBB stained 2DE of a human serum sample using our suggested method (A) and “tube gel” standard 2DE method (B). There is significant number of protein spots on gel (A) compared to the standard (B).

We have also compared human sera analyzed using O’Farrel tube gel method with our suggested 2DE method. Results clearly show that our method gave a better protein profile compared to O’Farrel tube gel method performed under our lab conditions (Fig. 4).

CONCLUSIONS

2-DE is one of the most useful methods of protein separation. Here we introduce a modified method of 2-DE in which isoelectric focusing is carried out in the first dimension using a vertical SDS-PAGE apparatus. Following focusing, each individual lane is excised and inserted into a specially fabricated vertical SDS-PAGE gel for the second dimension run. This modified version of IEF can be run in less than 2 hours and up to 10 samples can be analysed using only one IEF gel.

A similar method has been reported previously by *Robertson et al.*, in 1987 [5]. However our method suggests vast improvements over their method and they are as follows: (a) in our suggested 2-DE method, a larger sample volume can be loaded in the first dimensional gel (up to 100 μ L) compared to 50 μ L in *Robertson et al.*, method; (b) isoelectric focusing can be performed in less than 2 hours including pre-focusing compared to *Robertson et al.* method; (c) in our suggested method, a well is designed for loading the molecular weight marker by inserting a 2-cm long plastic piece prior to polymerization of the top gel portion (to seal the excised IEF gel lane). There is no loading well available for loading protein marker in *Robertson et al.*, method. Having protein marker in the second dimensional SDS-

PAGE gel is very useful in clinical studies, allowing protein spots to be analyzed based on their apparent molecular weight; (d) the IEF excised gel strip from the first dimensional IEF is polymerized to the second dimensional SDS-PAGE gel with the same ingredients that is used for casting the SDS-PAGE gel to avoid possible air bubbles and/or protein diffusion (this is not done in the *Robertson et al.* method, where they specifically state that agarose was not used to seal the gel); (e) In our suggested method, there is no need for fixing or staining the first dimensional IEF as carried out in *Robertson et al.*, method. In their procedure the staining process takes more than 2 hours; (f) In *Robertson et al.*, method, since proteins in the first dimensional IEF are fixed, a high voltage (150 V) is used for running the second dimensional gel [5], which necessitates a refrigerated water bath to cool the system. However, our suggested method eliminates the high voltage run for the second dimensional SDS-PAGE gel (constant current, 25 mA which is about 50 V).

The disadvantage of O’Farrel method are: (a) it is relatively difficult to cast the gel; (b) it is difficult to extrude the tube gel following IEF run; (c) there is the possibility of gel breakage during gel extraction; (d) the gel sometimes slides out of the glass tube during an overnight run; and, (e) the sample run takes 16 hours. In the flatbed IEF method, there is the possibility of sample leakage in between the comb and the gel. With regard to IPG gels, the procedure requires expensive instrumentation and material. Rehydration of IPG gel strips requires an overnight incubation step. Strips have

to be incubated with antigen. It takes about nine hours to run and uses high voltage to run (up to 7000 volts) [3,13]. High voltage usage, long duration IEF run and/or focusing time in most of the available IEF methods are some of the disadvantages which our method obviates and thus offers a simple way to carry out 2-DE [3,4].

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