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Full Length Research Paper

Developing High-Resolution Horizontal Native PAGE for Proteins: Implications for Analytical Applications in Biotechnology

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Horizontal PAGE is used to analyze negatively and positively charged proteins in a single gel. In the current study, we have developed a simple, user friendly and versatile horizontal PAGE apparatus to run native polyacrylamide gel. We have rigorously tested the ability of the gel system to resolve positively and negatively charged proteins or complex biological samples. The native PAGE resolves proteins based on their charge/mass ratio and a linear relationship was observed between migration (Rf) and the mass / charge ratio of different standard test proteins. Analysis of Bacterial lysate on horizontal native PAGE in 1st dimension and vertical SDS-PAGE in 2nd dimension gives discrete spot pattern on gel. In this current form, the 2-D pattern is not complementary to usual 2-D gel (IEF followed by SDS-PAGE) but it is useful to standardize many factors to observe proteome differences. Besides these analytical applications, designed apparatus can be used to purify native proteins in bulk for biochemical characterization, antibody development, activity assay for pilot studies.

Keywords: Protein, Horizontal apparatus, Polyacrylamide gel, Charge, Mass, Electrophoresis.

INTRODUCTION

Separation and analysis of macromolecules are the key step for success of biochemical and molecular biology experiments. Complex protein mixture or nucleic acid solution can be easily analyzed in a polyacrylamide or agarose gel respectively. Macromolecules have characteristic electrical and physio-chemical properties (charge, mass and 3-D conformation) which are exploited during gel electrophoresis (Raymond, Nakamichi et al. 1962; Zeiger, Salomon et al. 1972). Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) separates a protein mixture based on their relative molecular mass whereas native PAGE resolves protein based on the charge/mass ratio . Usually a vertical gel apparatus is used to cast and run polyacrylamide gel. Vertical gel apparatus contains anodic electrode in the lower tank and cathodic electrode in the upper tank. Both electrodes are connected by a polyacrylamide gel, sandwiched between two glass plates. Once electric fields is applied, current runs between two electrodes through polyacrylamide gel and gives a driving force for charged protein molecules (Knauf, Proverbio et al., 1974). The vertical gel system is good to analyze negatively charged protein (native or SDS bound) or positively charged proteins by reversing electrode polarity; but it is not possible to analyze proteins of both charges in the same gel. Later on, horizontal gel apparatus seems suitable to analyze native proteins of both charges in the same gel. In this apparatus, protein loaded in the middle of the gel moves towards opposite charge electrode on the basis of the charge/mass ratio.

Horizontal gel apparatus, developed to run DNA/RNA in agarose gel is not appropriate to cast polyacrylamide gel. A number of horizontal gel apparatus has been developed to cast and run polyacrylamide gel (Su, Wang et al. 1994; Gorg and Weiss 1999; Kim, Yokota et al. 2000; Huang, Zhou et al. 2004; Izzo, Costa et al. 2006). The horizontal PAGE apparatus developed by Su et al., 1994 to run native polyacrylamide gel is easy to fabricate

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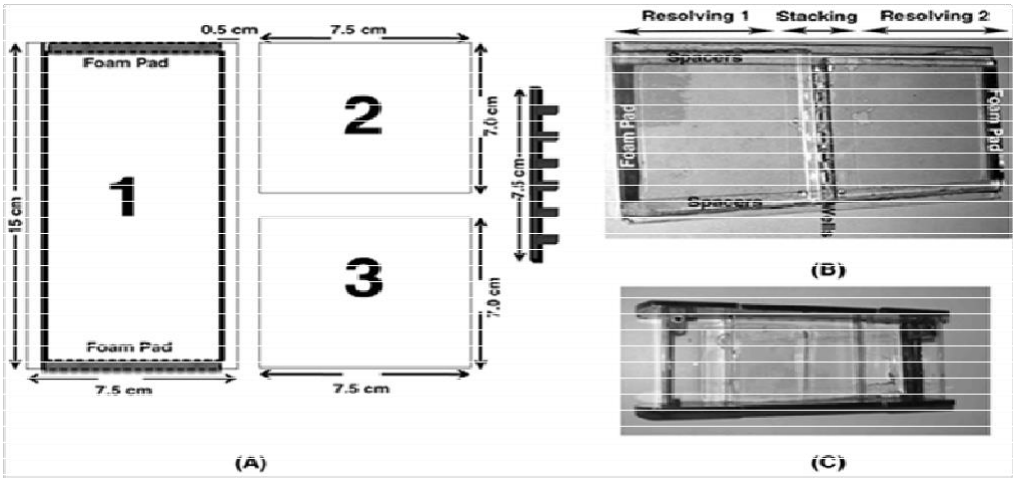

Figure 1: Design of Native Horizontal Apparatus (A) Dimensions of different glass plates used as components of Gel cassettes. (B) Picture of fabricated glass plate and comb used to cast gel in cassette. All these components are fabricated locally with the help of glass cutter and then assembled in our lab. (C) Picture of complete horizontal gel apparatus with gel assembled gel cassette placed in electrode chamber.

in the lab but gel casting and running is time consuming and not user friendly (Su, Wang et al. 1994). Recently, the design proposed by Izzo et al, 2006 has made significant advancement in gel casting; added a number of features such as casting gels of different length and breadth (number of well). This horizontal PAGE apparatus requires specialized glass plate with teeth to cast gel but the fabrication of glass plate needs assistance of specialized personnel (Izzo, Costa et al., 2006). In addition, most of the earlier design doesn't allow user to cast resolving and stacking gel together, which might be responsible for the poor resolution.

In the current study we have put effort to design an advanced version of the native horizontal PAGE apparatus. The design proposed by Su et al., 1994 was used as a base model to develop more user friendly, less time consuming and cost effective apparatus. The horizontal native PAGE separates protein mixture with high resolution and protein migration (R_f) correlates well with mass/charge ratio. Our design is suitable to perform 2-D in conjugation with SDS-PAGE to separate and analyze complex biological samples. Besides analytical applications, it can be exploited to perform western blotting, native preparative gel to purify proteins in bulk for activity assay, antibody development etc. Moreover, our design doesn't need any specialized fabrication and it allows user to cast stacking and the resolving gel together.

MATERIAL AND METHODS

Acrylamide, Bis-acrylamide, Ammonium per sulphate (APS), N,N,N',N'-tetramethyl-ethane-1,2-diamine (TEMED), Sodium dodecyl sulfate (SDS), Butanol, Agarose, Sucrose, Bromophenol blue, Methylene blue, Coomassie brilliant blue R250, Methanol, Glacial Acetic acid, Glycine, Tris-HCl, NaOH, HCl, ß- mercaptoethanol (β-me) were purchased from Sisco research laboratories (SRL) pvt, ltd. Tween-80, Tritonx-100, RNase were procured from Himedia. Bovine serum albumin (BSA), Cytochrome C, Human Hemoglobin were from Sigma.

Design of the Gel Cassette

The horizontal native PAGE cassette and comb is fabricated as shown in Figure 1A and consists of 3 glass plates. Large plate (no.1, Figure 1A) is made up of 3mm thick glass with dimensions 15x7.5 cm and other two identical small plates (Plate no. 2 and3, Figure 1A) are made up of 2mm thick glass with dimensions 7.5x 7 cm. A 5mm wide and 2mm thick glass slide has been stick with araldite on both sides of the large plate to give in build spacers. A 2mm thick foam pad (impregnated with agarose to avoid leakage) is stick to both ends of the large plate to use as a sealant. After assembling the glass plates with the help of binder clips, both glass plates leave a 1 cm gap between them to place comb. Comb is fabricated with 2mm thick glass slide containing 6 vertical glass teeth of dimension 5x2 mm.

Casting of the Horizontal Native Polyacrylamide Gel

Gel cassette is assembled by placing both small glass plates on large plate and held together by binder clips so that it leaves a 1cm gap between them to place comb.

Before pouring resolving gel, leakage of the cassette is checked. Place the whole cassette in the vertical position and 8% acrylamide solution is prepared as per published formula (Wang and Pan 1991) and poured into the cassette through 1cm gap up-to ¾ height of the glass plate (glass plate no. 2). A thin layer of water equilibrated butanol is over-lay on top of resolving gel. Once the resolving gel is polymerized, gel cassette has been turned up-side down and again repeats the same processes as described before to cast the resolving gel on the turned side of the glass plate (glass plate no. 3). Once the casting of resolving gel is over, surfaces of the both resolving gel is washed with water thoroughly and whole gel cassette has been placed on a horizontal plane. A 5% stacking gel is poured through 1cm gap between glass plates and comb has been placed to cast wells.

Sample Preparation

Protein samples are mixed with 5x loading dye, containing 40% sucrose, 10% bromophenol blue (BPB), and 10% methylene blue (MB). BPB is an anionic dye and used to monitor mobility of proteins on the anodic side whereas MB is a cationic dye to track the movement on the other side (cathode) of the gel.

Electrophoresis of proteins in Horizontal Native PAGE

Once the stacking gel is polymerized, comb is removed and wells are washed with water or 1x native Tris-Glycine running buffer (pH 8.3). After removing binder clips and foam pads, gel cassette has been placed in the horizontal electrophoresis apparatus. Electrode chambers are filled with chilled 1x native Tris-glycine running buffer to the level which is just enough to reach up-to glass plate level. Load the sample (up-to 20µl) into the wells and electrophoresis is performed at constant 100V in a cold room.

Staining and Destaining of the Horizontal Native Gel

After electrophoresis is over, gel is removed from the cassettes with the help of scalpel and stained with coomassie brilliant blue. The whole process of staining and destaining of gel completes in less than 3 hrs.

Preparation of E. Coli Cell Lysate

5 ml O/N grown *E.Coli* culture is re-suspended in 2ml lysis buffer containing 100mM Tris pH 6.8, 1mM EDTA, 1%TritonX-100. Cells are sonicated for 20 sec pulse at 20% energy output and resulting lysate is centrifuged at 12000 rpm for 10mins to get clear supernatant. 2ml Bacterial lysate is precipitated by 10% TCA, resulting precipitate is re-dissolved in 50µl lysis buffer and loaded on 8% native horizontal PAGE.

Preparation of Plant Latex Extract

Plant latex from calotropis procera is prepared as described previously (Narain, 2010). 200µl latex is clarified by 10% TCA precipitation, re-dissolved in 20µl lysis buffer and resolved on 8% native horizontal PAGE.

2-Dimensional Gel Electrophoresis

Bacterial lysate is allowed to run on 8% native PAGE in horizontal apparatus until dye reaches to the end of the gel. Sample strip from both side (anodic and cathodic) is cut from native PAGE with the help of a sharp razor and soaked into de-naturation buffer (100mM Tris pH 8.8, 3% SDS, 5mM ß-mercaptomethanol, 1mM EDTA) at 60°C for 2 hrs. A 12% SDS-PAGE is prepared without stacking gel and the sample strip is placed on top of resolving gel. The gap between resolving gel and sample stripe is filled with 5% stacking gel. Once the stacking gel is polymerized, SDS-PAGE is allowed to run at constant voltage (initially 100V and then afterwards at higher voltage) up-to the end of the gel. Gel is removed from the glass plates and developed by coomassie staining and image is analyzed by Image J 1.43u (Girish and Vijayalakshmi 2004).

RESULTS

Design of Horizontal Native PAGE Apparatus

Design of the horizontal native PAGE apparatus is shown in Figure 1. The gel cassette consists of 3 glass plates which can be assembled without much effort. Presence of foam pad on the bigger glass plate gives in-build sealing to facilitate easy and less time consuming casting. Moreover, the current gel cassette can be easily accommodated into a commercially available mini-horizontal gel apparatus.

Characterization of Protein Separation in a Native Horizontal Apparatus

Native gel resolves protein based on the charge/mass ratio and native conformation. A number of physical and chemical parameters of horizontal gel system (length and thickness of resolving and stacking gel, temp etc.) need optimization to achieve better resolution. We have

Table I: Biochemical Properties of Standard protein at running buffer pH 8.3.

S/No	Protein	Mol. Weight (Kda)	pI	Net Charge	^Rf Value ± SD
1	BSA	66	5.6	- ve	0.54 ± 0.0719
2	RNase	15	8.6	+ve	0.265 ± 0.0187
3	Cytochrome C	12	10.0	+ve	0.6433 ± 0.0568
4	Human Hb	68	7.1	-ve	0.036 ± 0.0
5	Plant Protease	25.7	9.5	+ve	0.56 ± 0.0707

^Rf is calculated by the equation,
Rf=Distance migrated by protein/distance migrated by tracking dye.

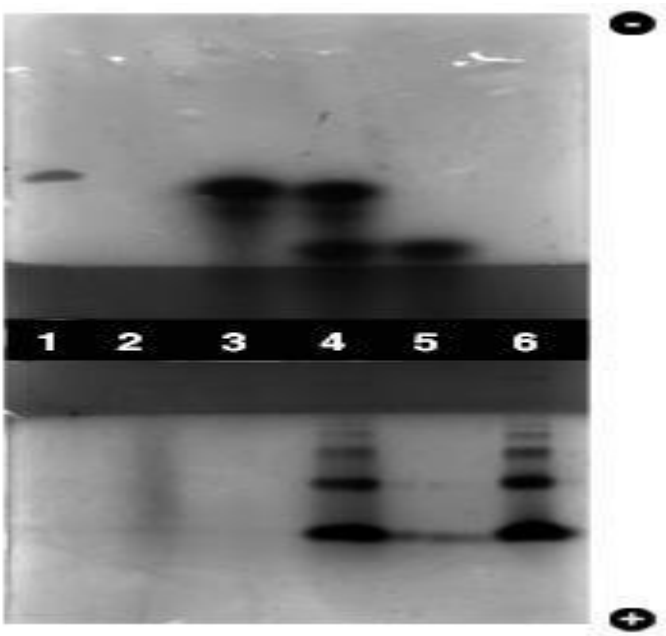

Figure 2: Resolution of Standard positively and negatively charged proteins in the native horizontal gel system. Analysis of Plant Protease (Lane 1), Human hemoglobin (Lane 2), CytC (Lane 3), Mixture of all proteins except plant protease (Lane 4), RNase (Lane 5), BSA (Lane 6) on 8% native horizontal PAGE system. Standard purified protein solution (10mg/ml) is mixed with 5x sample buffer and 20 µl is loaded into separate lane and electrophoresis is carried out at 100V in chilled 1x Tris-glycine running buffer.

chosen a variety of purified standard proteins as a reference to establish these parameters. The biochemical properties of standard proteins used in this study are given in Table 1. As expected, gel system separates the proteins to form sharp and discrete bands on the gel (Figure 2). Next, we checked whether polyacylamide gel holds a linear relationship between the charge/mass ratio and relative mobility (R_f) of the protein. Rf of individual proteins is calculated by measuring the band distance from the middle of the well divided by the distance moved by dye front. The R_f Value of different proteins is listed in Table 1 and a plot of R_f with charge/mass ratio is given in Figure 3. Both parameters are linear over a range and hold the correlation for both positively and negatively charged proteins.

Analysis of Complex Biological Samples

Further, we have tested the ability of this gel system to resolve a mixture of proteins. For this purpose, we have prepared the mixture of standard proteins (BSA, RNase, CytC, Hb) and loaded into the native horizontal PAGE. We have kept plant protease separate from this mixture as it may cause degradation of other proteins. The native gel has resolved the mixture and the protein bands co-

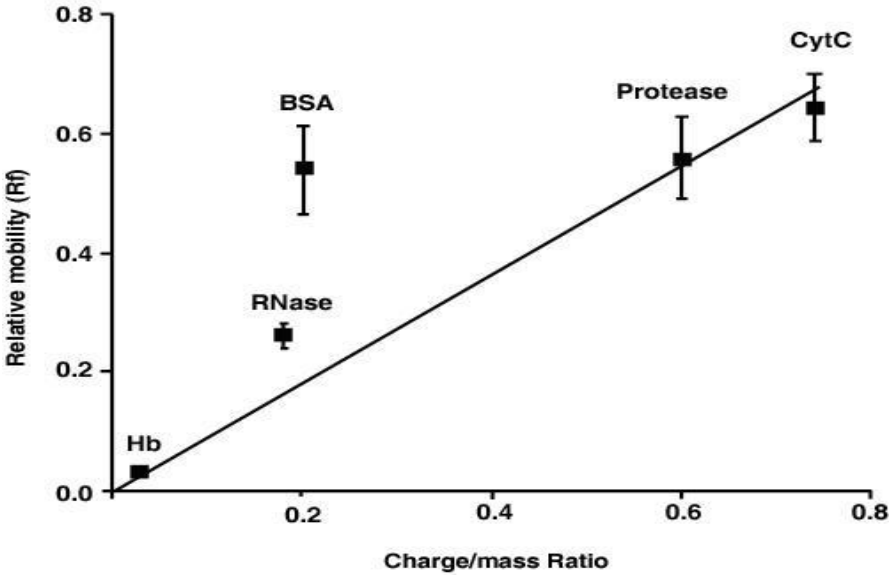

Figure 3: Correlation of charge/mass ratio and relative migration (Rf) of different standard protein. Standard purified protein solution (10mg/ml) is mixed with 5x sample buffer and 20 µl is loaded into separate lane and electrophoresis is carried out at 100V in chilled 1x Tris-glycine running buffer. Rf is calculated as a ratio of migration of the protein band from the middle of the well and migration of the dye front (mean±SD, n=7).

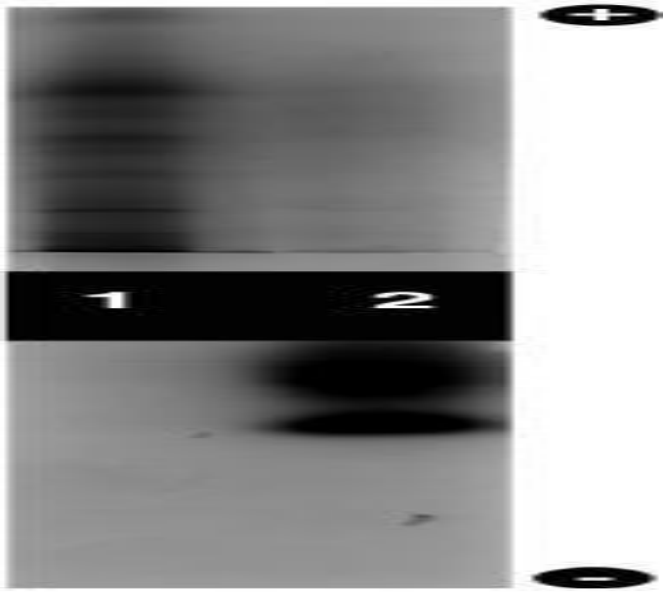

Figure 4: Analysis of Complex Biological Samples On Native Horizontal Gel System. Bacterial lysate (Lane 1) or plant latex (Lane 2) is prepared as described in method section and resolved on 8% native horizontal gel system.

Migrate with their respective standard proteins. This indicates, the presence of different protein together in the sample doesn't affect the resolution power of the gel (Figure 2, lane 4). We further wanted to test the ability of this gel system by analyzing complex biological samples. E. coli lysate and plant latex are resolved on horizontal native PAGE at 100V for 4.5 hrs in cold room. The horizontal gel system has separated both biological samples with a high resolution to give discrete sharp bands corresponding to proteins of different charge/mass ratio (Figure 4).

A 2-D gel electrophoresis is performed to exploit

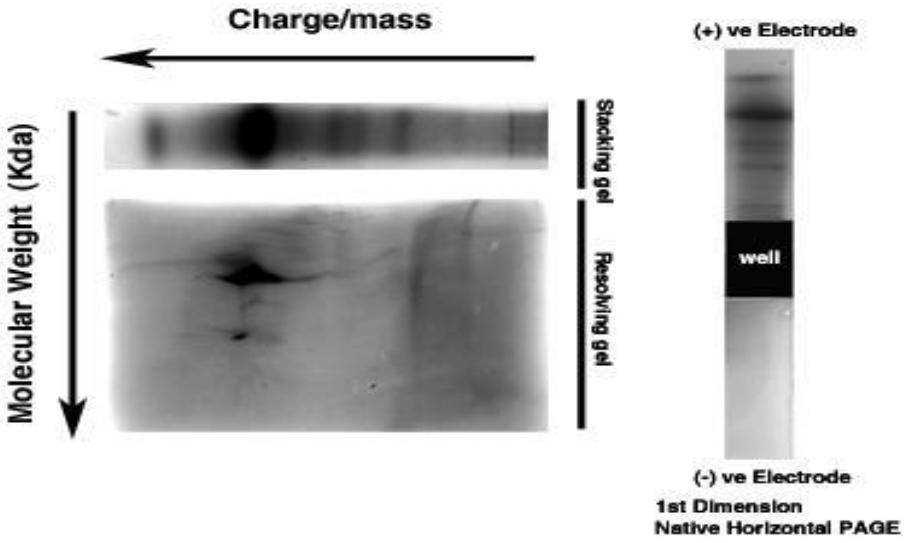

Figure 5: Two Dimensional (2-D) Resolution of Bacterial lysate. Bacterial lysate is prepared as described in method section and resolved on 8% native page (Ist dimensions) in horizontal apparatus and then anodic stripe is cut with a sharp rajor and resolved further on 12% SDS-PAGE in a vertical apparatus. Gel is stained with coomassie and the image is analyzed by Image J 1.43u.

different physio-chemical properties of macromolecules. One of the popular combinations is pI (Isoelectric focusing) and mass (SDS-PAGE) to resolve complex biological protein sample. But iso-electric focusing is a costly technique and require specialized instrument to perform(O'Farrell 1975). Thus we also tested the performance of horizontal gel system in the 2-D gel analysis of complex biological samples, we resolved bacterial and plant latex in native horizontal PAGE apparatus (1-D) and SDS-PAGE (2-D) in a vertical apparatus as described in material and method section. The gel developed by coomassie staining exhibits a discrete spot pattern on the gel (Figure 5). An image analysis by Image J 1.43u has identified a number of protein spots. We haven't been able to detect protein band from the cathode side of the native horizontal gel and probably it requires more standardization. One major reason of low protein spots in 2nd dimensions would be the diffusion of protein during de-maturation step and low level of SDS derivatization.

DISCUSSION

Development of high resolution native horizontal PAGE is the main focus of the current study. A good gel system should be cheap, user friendly, reproducible and of high resolution. The gel cassette used in this study is made up of glass plates; fabricated locally with the help of glass cutter. It is cheap (each set cost ~$4-5) and the fabrication can be reproduced in any other laboratory without much effort. Use of stacking gel to compress the protein band is very popular in the vertical PAGE system but it was never tried in a horizontal PAGE system. The commercially available pre-cast clean polyacrylamide gel from Amersham biosciences has wells in the middle, contain stacking gel only on one side of the well and it runs on Multiphor II PAGE systems. This gel system gives very high resolution but only suitable to analyze acidic proteins (Heukeshoven and Dernick 1992). Besides this gel no other gel system has been developed so far where a stacking gel has been tried on both sides of the well to compress both acidic and basic proteins. In our study stacking gel is present on both sides of the well and protein bands are compressed (Figure2). Although the electrophoretic mobility theory of ions (Tris-protein-glycine) supports stacking of negatively charged proteins but we are not sure if the similar mechanism is operating for the compression of positively charged proteins as well. Also, we have not tested extensively how stacking gel effects protein band compression. But provision of the stacking gel casting gives the user a freedom to try out and achieves better results.

In our extensive testing with the standard purified proteins, a mixture of standard protein or complex biological samples; gel system has resolved the sample with high efficiency on both sides of the well (positively and negatively charged) to give discrete compact bands (Figure 2). In addition, the protein migration (R_f) and charge/mass ratio are well correlated with each other (Figure 3) and give a straight line (R^2=0.951). These two parameters are only tested with 5 standard proteins (BSA, RNase, Hb, CytC, Plant Protease) but the curve based on such a small sample number is not helpful to predict charge/mass of an unknown protein. This requires extensive calibration of whole gel length with a

Large number of protein samples with varying charge/mass ratio which is not the focus of the current study.

The results depicted in Figure 5 are an initial attempt to test the ability of this system for 2-D gel analysis of complex biological sample. In a 2-D gel analysis of bacterial lysate, we have seen many protein spots identified by ImageJ1.43u. A number of spots are missing in comparison to expected number of spots (44 spots) on a Virtual 2-D gel of E.Coli lysate. At this moment our gel system doesn't have the potential to give results comparable to existing 2-D gel electrophoresis (pI/SDS-PAGE). To exploit this system as a robust alternative to typical 2-D gel needs more rigorous efforts. One can easily expect better resolution with gradient SDS-PAGE in 2nd dimension which have not been tested so far.

In a typical 2-D gel analysis, mostly researcher exploits two properties such as isoelectric point (pI) and molecular mass of the protein (O'Farrell 1975). This combination gives very high resolution and be a standard technique to see minor changes in proteome of an organism under certain experimental or biological conditions. The technique requires costly instrumental setup and consumables. Thus it cannot be run on multiple occasions to standardize different experimental parameters. Hence our gel system is a good starting point to standardize various experimental parameters to observe results.

Moreover, we have put efforts to testify many features of the gel system such as resolution power to separate protein mixtures as well as complex biological samples such as bacterial lysate. In conclusions, we have developed an improved version of the native horizontal gel electrophoresis system with a number of features which facilities easy casting and running of both negatively and positively charged protein in the same gel.

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