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Influence of Gel Dimensions on Resolution and Sample Throughput on Two-Dimensional Gels

KiBeom Lee[1]*, KyungBae Pi[2], and Hong-Gu Lee[3]*

[1]*Department of Biotechnology, SongDo TechnoPark, 7-50 Songdo-Dong, Yeonsu-Gu, Incheon, Korea 406-840; fax: 82-32-260-0769; E-mail: klee02@empal.com*
[2]*Research Institute, AdipoGen, Korea University, College of Life Science and Biotechnology, Rm#538, 5-Ka, Anam-Dong, Sungbuk-Gu, Seoul, Korea 136-701*
[3]*School of Agricultural Biotechnology, Seoul National University, Seoul 151-742, Korea; fax: 82-2-875-7340; E-mail: hglee66@hotmail.com*

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Abstract—To achieve high throughput and economical format of 2-D PAGE, comparison between gel size and resolution was conducted on human breast carcinoma cell line (MCF-7/AZ) proteins. SDS gel length showed a weaker influence of separation length on resolution in the second dimension, and there was little benefit of separation distances greater than 15 to 19 cm. IPG strip separation distances were very important with dramatic increase in resolution of longer gels compared with smaller gels, and maximal resolution was obtained using 18- and 24-cm IPG strips. Loading optimal amount of proteins on 2-D gels can also increase the number of detected spots. Therefore, taken together, compromise 2-D gels are crucial for higher capacity and higher throughput.

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For the past several decades high-resolution two-dimensional polyacrylamide gel electrophoresis (2-D PAGE) has been the technique for separating many types of proteins [1-4]. The technique involves the separation of proteins by charge in the first dimension and by molecular weight in the second dimension. The traditional 2-D PAGE technology relied on the use of carrier ampholytes to establish the pH gradient [2, 5, 6], but this technique has proven to be difficult in the hands of many because of intensive labor and the lack of reproducibility created by uncontrollable variations in the batches of ampholytes used to generate the pH gradients.

Recently, the introduction of immobilized pH gradient (IPG) technology [7-9] has made possible obtaining more reproducible focusing patterns and improving the separation of whole cell proteome. Therefore, the technique plays a central role in "proteomics", an area of functional genomics that deals with the global analysis of gene expression at the protein level. The best protein separation is obtained when performing isoelectrofocusing (IEF) on large gels. However, the application of 2-D PAGE for high-throughput sample separation is limited by time and cost when large gels are used. Small format gels can simplify the 2-D process and decrease the overall time required. However, small gels yield poorly resolved protein spots.

Consequently, some problems in 2-D IPG gels still remain to be solved, such as speed, cost, resolution power, and high-throughput of 2-D PAGE. The aim of this work was the use of an efficient 2-D gel system to minimize time and cost expenses during experiments. Therefore, to render this system more accessible, we explored whether current 2-D gel system could be simplified using commercially available gel units.

MATERIALS AND METHODS

Electrophoresis equipment and chemicals. All reagents used were of analytical grade or better and all buffers were prepared using MilliQ water. The Protean® IEF cell,

Abbreviations: 2-D PAGE) two-dimensional polyacrylamide gel electrophoresis; IEF) isoelectrofocusing; IPG) immobilized pH gradient.

* To whom correspondence should be addressed.

Protean® II Xi and Criterion dodeca cells for SDS-PAGE, bromophenol blue, agarose, Tris, acrylamide, bis-acrylamide, ammonium persulfate, TEMED, and 7-cm IPG strips (pH 4-7) were purchased from Bio-Rad (USA). An Ettan Dalt SDS-PAGE system, 11-, 18-, or 24-cm IPG strips, carrier ampholytes pH 4-7, 3-10NL, urea, Chaps, mineral oil, glycerol, dithiothreitol (DTT), and 2-D Quant protein assay kit were purchased from Amersham Biosciences (USA). Thiourea and iodoacetamide (IAA) were purchased from Sigma-Aldrich (USA).

Sample preparation. Cultured cells of human breast carcinoma cell line, MCF-7/AZ, were grown *in vitro* in a 37°C incubator with a 5% CO_2-95% air atmosphere in a 1 : 1 mixture of Dulbecco's modification of Eagle's medium/Ham's F-12 (Mediatech Inc., USA) supplemented with 10% fetal bovine serum (Atlanta Biologicals, USA). Prior to protein extraction, 80-90% confluent cells in T75 flasks were washed three times with phosphate-buffered saline containing protease/phosphatase inhibitors (0.15 mM phenylmethylsulfonyl fluoride, 2 mM Na_3VO_4, 50 mM NaF, 1 μg/μl leupeptin, 1 μg/μl pepstatin-A) at 0°C. Cells were immediately lysed *in situ* on ice with lysis buffer containing 9 M urea, 2 M thiourea, 4% Chaps, 100 mM DTT, 5 mM EDTA, 0.2% IPG-buffer (3-10L), and protease/phosphatase inhibitors (see above), pH 8.0, for 10 min. The flasks were scraped to remove remaining insoluble material.

The extracted samples were transferred to a plastic centrifuge tube and sonicated on ice with a probe-tip sonicator using four 2 sec bursts with 4 min pauses between sonication cycles to prevent overheating. The supernatant was collected by centrifugation at 40,000*g* for 30 min at 4°C. The pellets were resuspended in a small volume of lysis buffer, sonicated, and centrifuged as above. This supernatant was combined with the initial supernatant, and represented the soluble component of approximately 10^7 cells/ml at a protein concentration of about 1.0 mg/ml. The actual protein concentrations of the supernatant and pellet were determined, and samples were stored as aliquots at −80°C until needed.

Two-dimensional gel electrophoresis. For 2-D gels, samples were diluted into IEF buffer containing 9 M urea, 2 M thiourea, 4% w/v Chaps, 0.8% v/v carrier ampholytes (pH 4-7 and pH 3-10NL), 1% w/v DTT, and

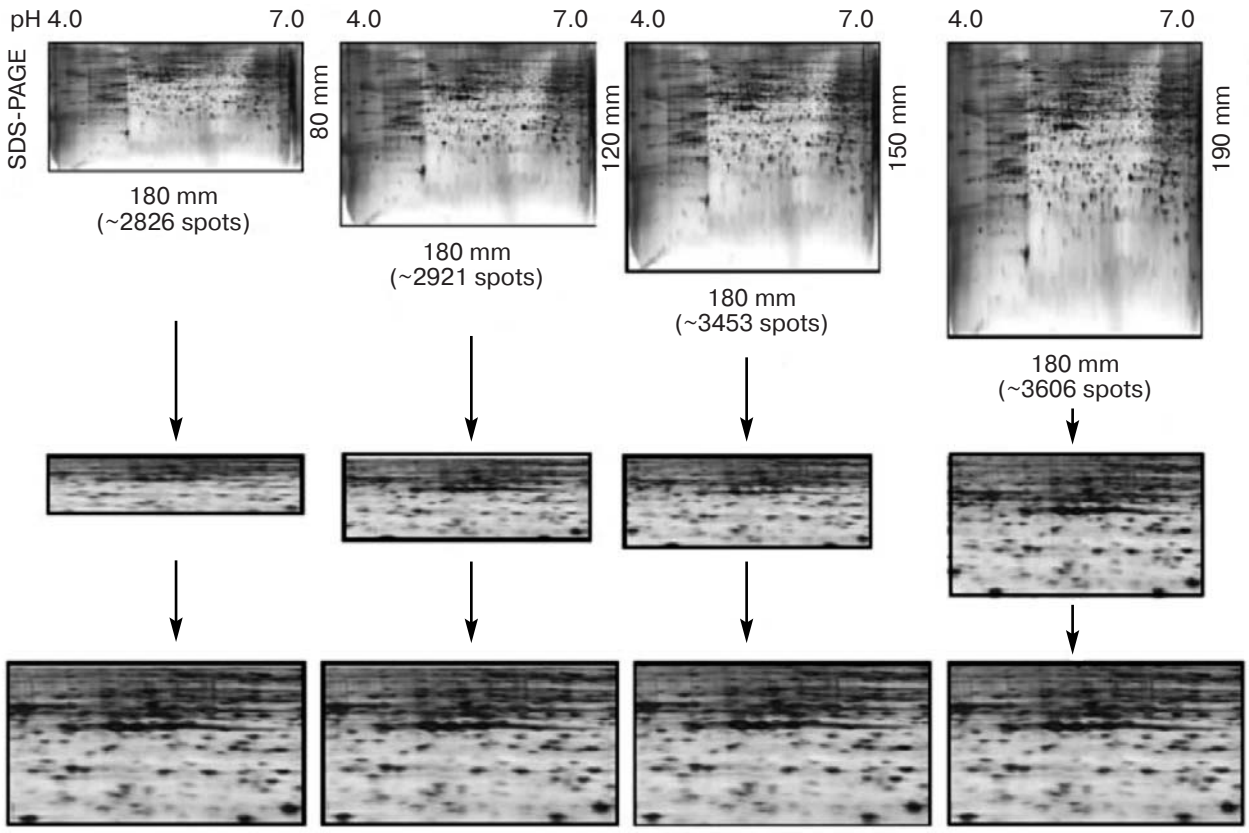

Fig. 1. Comparison of the resolving power of 2-DE patterns using different length SDS gels. Fifty micrograms of proteins was focused on 18-cm pH 4-7 IPG strips. After IEF, all samples from IPG strips were run on 8-, 12-, 15-, and 19-cm 10% Tris-Tricine SDS gels followed by silver staining. The total number of spots identified using Melanie 4 software is shown (upper panel). The middle panel shows equivalent molecular weight range for different sized gels. Lower panel: equivalent molecular weight range for different sized gels was stretched to equal image sizes for direct visual comparisons of spot resolution.

a trace of bromophenol blue to yield the desired protein amount in a volume that could be adsorbed by the IPG strip used. This sample in buffer was also used to rehydrate 7-, 11-, 18-, or 24-cm IPGs for 12 h at 50 V, incubated at room temperature. All IEF was performed using the Protean® IEF Cell and its 7-, 11-, 18-, and 24-cm IEF tray. The following voltage program was used after the 12-h rehydration: 1) 7-cm IPG strips; linear ramp to 250 V over 15 min, followed by linear ramp to 4000 V over 2 h, then 4000 V constant for 5 h, for a total of 24,000 V·h; 2) 11-cm IPG strips; linear ramp to 250 V over 15 min, followed by linear ramp to 8000 V over 2.5 h, then 8000 V constant for 4.3 h, for a total 44,000 V·h; 3) 18- or 24-cm IPG strips; linear ramp to 250 V over 15 min, followed by linear ramp to 10,000 V over 3 h, then 10,000 V constant for 6 h, for a total of 75,000 V·h. Focused IPG strips were stored at –80°C before equilibration and application to SDS-PAGE.

Equilibration and second dimension SDS-PAGE. After IEF, IPG strips were equilibrated using 6 M urea, 50 mM Tris-HCl, pH 8.0, 30% glycerol, 2% SDS, and 30 mM DTT for 10 min followed by 6 M urea, 50 mM Tris-HCl, pH 8.0, 30% glycerol, 2% SDS, and 5% iodoacetamide for 10 min. The IPG strips were then embedded in hot 0.5% agarose (~70°C) containing bromophenol blue on top of 10% polyacrylamide gels. The second-dimension gel electrophoresis was subsequently performed on different sizes of 10% SDS-polyacrylamide gels. Separation was performed at 100 mA constant current with external cooling until the tracking dye migrated to within 1 cm of the bottom of the gel.

Staining of proteins. Upon completion of 2-D SDS-PAGE, gels were stained using the SilverQuest (Invitrogen, USA) stain as described by the manufacturer. Spot detection and analysis were performed using the Melanie 4 software (Genebio). All experiments depicted were performed at least in triplicate. Representative single gel images are shown in the figures.

RESULTS

Fixed IPG gel lengths versus varying SDS gel lengths. We investigated whether the gel dimensions affect how

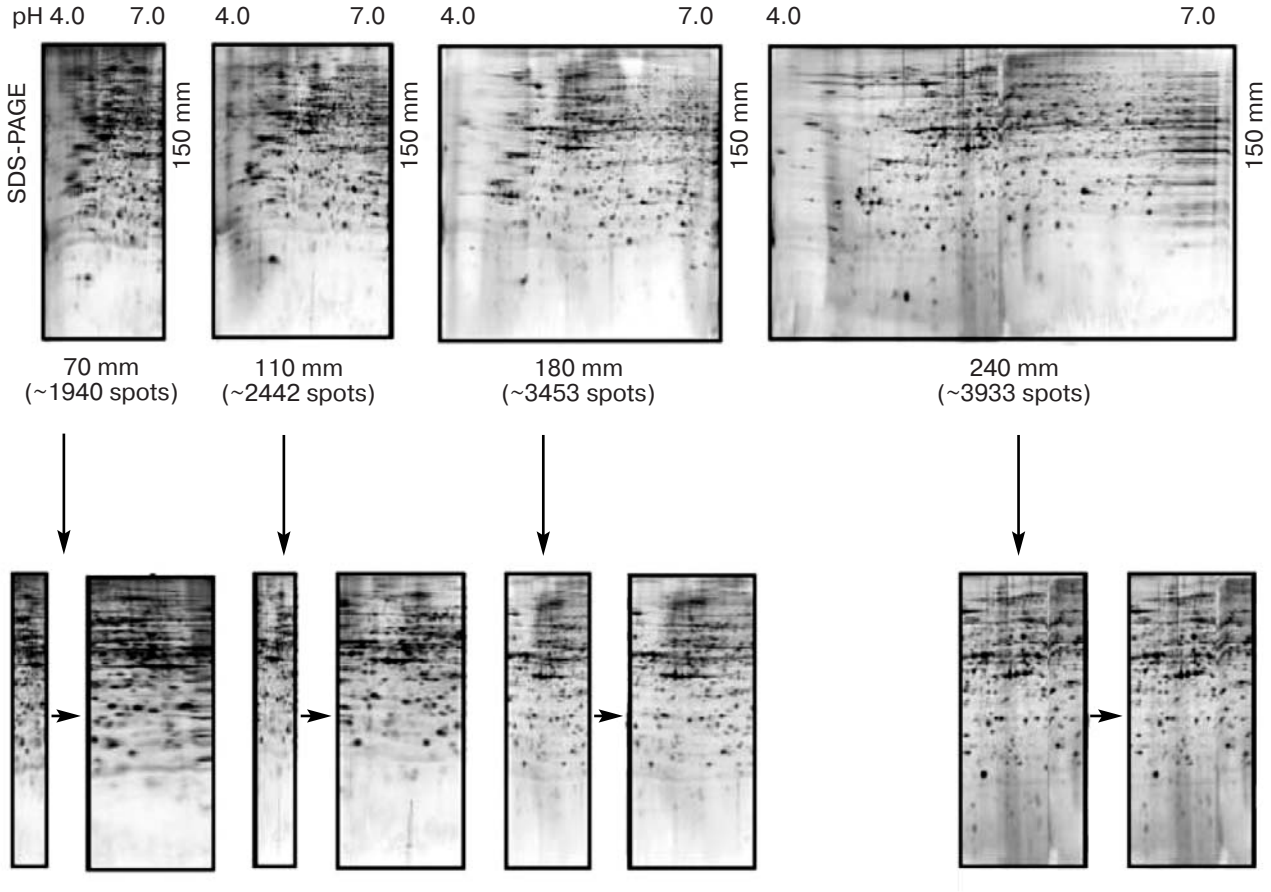

Fig. 2. Comparison of resolving power of 2-DE patterns using different length IPG gels. Fifty micrograms of proteins was focused on different pH 4-7 IPG strips. After focusing, the IPG strip was separated in the second dimension using 15-cm length IPG gels. The total number of spots identified using Melanie 4 software is shown (upper panel). Lower panel: equivalent pH range for different sized gels was stretched to equal image sizes for direct visual comparisons of spot resolution.

many proteins can be revealed in proteomic research. With the first concern, we utilized 18-cm IPG (pH 4-7) in the first dimension. The pH region chosen is the most highly clustered for human breast carcinoma cell line (MCF-7/AZ) proteins. In the second dimension, four different height gels (8-, 12-, 15-, and 19-cm) were run for each 18-cm IPG in order to compare the resolution of second SDS page (Fig. 1). Altogether, 50 μg of proteins were analyzed by the gels. Differences in the protein spots were apparent among the gels and the number of spots detected in 8-, 12-, 15-, and 19-cm length SDS gels were 2826, 2921, 3453, and 3606, respectively. It was clear that partially overlapping spots were contained in 7- and 12-cm gels. One option to reduce the problem of overlapping or superimposed protein spots is to increase SDS gel format. It seemed to be interesting that resolution dramatically increased as SDS gel lengths were increased from 12 to 15 cm. Despite the apparent differences in the SDS gel length, there appear to be very few differences in the number of spots detected for 15- and 19-cm SDS gels, supporting the idea that 15-cm SDS gel is an effective gel size on resolution and sample throughput. Longer gel separation distances are likely to increase the total number of analyses per proteome, but the benefits of increased SDS gel separation distance are ambiguous at a certain level. Because studies showed that 15-cm SDS gel provided a powerful tool to isolate protein spots with a reduced electrophoresis time, we decided to utilize 15-cm SDS gel for all subsequent IPG gel length experiments.

Varying IPG gel lengths versus fixed SDS gel lengths. In the experiment shown in Fig. 2, the second concern was the effects of focusing length while keeping SDS gel lengths fixed at 15-cm. To address this issue, subsequent 7-, 11-, 18-, and 24-cm IPG gel lengths were used to compare the number of spots. Altogether, the amount of protein loaded onto each IPG gel length was 50 μg. Using this protocol, the number of spots detected in 7-, 11-, 18-, and 24-cm IPG gel lengths were 1940, 2442, 3453, and 3933, respectively. We found that relative separation of spot overlapping was significantly dependent on IPG gel lengths. The resolution of proteins was raised up to 24-cm in the first dimension. These high levels of resolution may be explained by the fact that increasing IPG gel length increases separation of individual spots, and apparently single spots divide into two or more protein spots. The short IPG gels did not seem to provide enhanced sample entry and resolution of proteins.

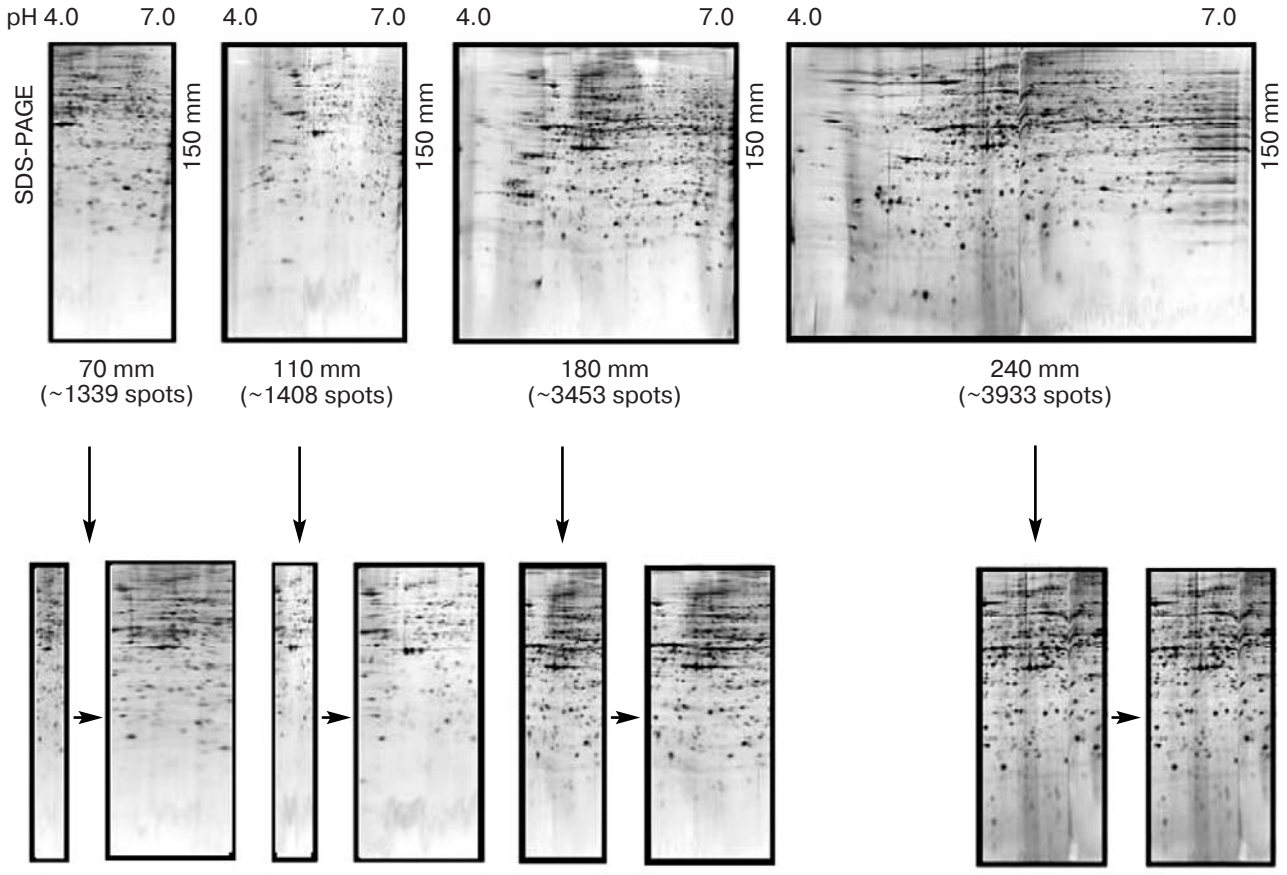

Fig. 3. Comparison of resolving power of 2-DE patterns using different protein loads (upper panel). Lower panel: equivalent pH range for different sized gels was stretched to equal image sizes for direct visual comparisons of spot resolution.

Next we investigated the effect of sample loading amount. When the same loading amount was used for each IPG gel length, we found overlapping or superimposed protein spots in the 7- and 11-cm IPG gel lengths. These results suggest that the number of spots obtained from 50 μg of protein is not saturated in small gels. Therefore, in the next experiment, in order to obtain good protein resolution on 2-D gels, optimal loading levels were evaluated. In order to decrease the effect of overloading amount on small gels, we adjusted the amount of proteins. We compared the systematic evaluation of IPG gel length while keeping SDS gel lengths fixed as 15-cm (Fig. 3). The spots detected by the 7-, 11-, 18-, and 24-cm IPG gels were as following: 1339 on 7-cm strip (protein 12.5 μg), 1408 on 11-cm strip (protein 12.5 μg), 3453 on 18-cm strip (protein 50 μg), and 3933 on 24-cm strip (protein 50 μg). By thus eliminating the overloading amount in 7- and 11-cm IPG gels, superimposed protein spots were also eliminated and resolution in 7- and 11-cm IPG gels was enhanced.

DISCUSSION

During IEF and second dimensional SDS running system, optimal resolution and the attainment of focusing of each protein are determined by many parameters (such as IEF and SDS gel lengths, and physicochemical characteristics of the proteins). To optimize protein resolution, we have investigated the effects of several parameters (SDS gel length, IPG gel length, and protein loading) that could affect protein profiling. To provide an improvement of the protein profiling strategies using 2-D gels, this study was performed using 7-, 11-, 18-, and 24-cm IPG gel lengths with SDS gel length ranges of 8-, 12-, 15-, and 19-cm. The main consideration is the effective gel size used. The smaller the gel size, the smaller protein numbers will be resolved, thereby substantially increasing the gel size needed to completely analyze a proteome. However, a major consideration for any comprehensive proteome analysis is the total time required to run a proteome. Increasing the gel size increases the run time for proteome separation as well as cost.

Therefore, a practical compromise between the number of proteins detected and run time as well as cost have to be considered. Considerable scope for improvement of results exists with combination of 18-cm wide by 15-cm long gel, and we would encourage the use of that size gels. In particular, using 15-cm SDS gels not only improved the resolution power and high-throughput of 2-D gel while saving time, but also staining costs. They could be conveniently mastered and applied because 18 × 16-cm glass plates are commercially available. When 7 × 16-cm and 11 × 16-cm gels were used with overloading amount, the number of proteins decreased moderately. The use of adjusted protein amounts in small gels enhanced resolution by minimizing protein diffusion and loss. Therefore, a good compromise between the IPG gel size and loading amount is needed.

In conclusion, we suggest an efficient 2-DE running protocol that not only improves resolution power and high-throughput of 2-DE but also can be easily performed and applied without negative effect. It thus offers a simpler, more rapid, effective, and economical format for the characterization and comparison of proteins under different experimental conditions.

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