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Original Research Article

Expeditious Molecular Weight Determination of Native Proteins by Transverse Pore Gradient Polyacrylamide Gel Electrophoresis Using the PhastGel System

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ABSTRACT

The Ferguson plot is well established method for the molecular weight determination of native proteins and protein complexes. The Ferguson plot requires running three or more gels of different polyacrylamide concentrations to determine the relationship between band migration and polyacrylamide gel concentration and using it solve for an unknown. Herein, we describe a new approach to a Ferguson plot that requires only one 8-25% gradient polyacrylamide gel, run as a transverse pore gradient, to provide the informational equivalent of 10-12 single concentration polyacrylamide gels. Using Cytochrome-C (14.4 kDa), E-GST (25 kDa), BSA (66 kDa) and BSA Dimer (132 kDa) as standards, we are able to determine the molecular weight of E-GST dimer with 9% experimental error in under 3 hours. This method has a significant advantage in reproducibility, efficiency, and cost effectiveness as compared to the standard method used for creating a Ferguson plot and is readily useable by any lab with a PhastSystem (GE LifeSciences).

Keywords: Electrophoresis, Ferguson plot, Molecular weight, Native, Polyacrylamide, Protein, Size

INTRODUCTION

Polyacrylamide Gel Electrophoresis (PAGE) is a well established technique that can be employed in molecular weight (MW) determinations of proteins (Laemmli, 1970). Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE) is used for MW determinations of denatured proteins. SDS-PAGE equalizes the charge of proteins (1.4g SDS/1 g protein) and denatures them into a globular shape, making molecular weight the only variable affecting a protein's movement through the gel. This allows accurate size determination using one gel for denatured proteins.

If a protein or protein complex is to have its molecular weight determined in native form, a far more laborious and time consuming method known as a Ferguson plot must be employed (Ferguson, 1964; Hendrick & Smith, 1968). This is because in addition to variable MW, proteins in native form have variable shape and variable charge that effectively skew any data

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gathered using the SDS-PAGE MW determination method. In order to overcome these variables, a series of NativePAGE gels of varying concentrations must be prepared. A Native PAGE gel is identical to an SDS PAGE gel except for the fact that no buffers are used while running and loading the gel, nor the gel itself contains any SDS. The logic behind the varying polyacrylamide gel percentages is that the relative mobility of proteins with respect to these percentages is dependent only on their MW, thus eliminating the variables of native charge and native shape so far as the protein standards have at least similar shape, similar charge and a similar degree of hydration as the protein whose MW is being determined (Andrews, 1981; Hames & Rickwood. 1998).

A method has previously been developed to avoid the need for multiple gels for a Ferguson plot (Rematal & Babul, 1988). This method very simply employs one gradient gel turned 90 degrees to create a transverse pore gradient allowing the study of protein migration over a range of gel concentrations using only one gel. Transverse pore electrophoresis is more generally used for DNA analysis in various forms (Hou, 2018). Furthermore, although the transverse pore gradient gel method has previously been used in the PhastSystem, they proved this method works with a DNA Ladder as a standard and not protein (Buzas et al., 1994). Herein, we describe an improved version of this method, in terms of efficiency, cost effectiveness, reproducibility and accuracy. Our method employs precast 8-25% PhastGels (GE Life Sciences) turned 90 degrees and run under native conditions using the PhastSystem (GE Life Sciences).

METHODS

Standard Proteins

Bovine Serum Albumin (66,000 Da), E-GST (25,000 Da) and Cytochrome C (14,400 Da). 4x Native Page Loading Buffer was also needed for protein preparation. All proteins were purchased from Sigma. The final concentration of each protein was prepared to be 10 ug/uL in 1X Native PAGE Buffer.

Apparatus, Accessories and Precast Gel

The PhastSystem apparatus (GE Life Sciences); a 8-well sample applicator; an 8-25 % polyacrylamide gradient PhastGel of 4 cm resolving path length containing 0.112 M Sodium Acetate, 0.112 M Tris, pH 6.0; and native buffer strips containing 0.88 M Alanine, 0.25 M Tris, pH 8.8 were needed.

Electrophoresis

The PhastGel gel was turned 90 degrees when placed into the apparatus with respect to the recommended orientation of the gel. It was important to place the gel in such a way as to maximise the resolving length of the gel. The contact surface of the buffer strips therefore did not need to be 100% in contact with the gel, so long as contact was kept constant throughout runs. With the gel oriented 90 degrees with respect to its recommended position, the protein loaded onto the gel ran at different gel concentrations (8%, 9%, 10%, etc) present throughout the gradient gel. The 8-well sample applicator was dipped into 2 µl of the standard proteins (10 µg/uL of each) which were dissolved in Native PAGE loading buffer, and set in place on the PhastSystem apparatus. When setting the applicator onto the apparatus, it was important to set it down gently and not let it impact the gel. Impact would result in a small amount of protein solution transferred onto the gel which entered the gel during the initial phase of electrophoresis. The initial phase uses voltage to saturate the gel with the buffer in the agarose

buffer strip and is not meant for protein separation. Ultimately this resulted in doublets of each protein band after staining, and made the gel almost impossible to analyse. If it so happens that the comb does impact the gel upon insertion into the apparatus, it is recommended to just remove the comb and run the gel as normal. This may generate some useful data and is preferable to just throwing the gel away.

Electrophoresis was conducted at 15 °C, 400 V. The initial, sample application and separation phases were set to 10 Vh, 2 Vh and 50 Vh, respectively. The progress of the separation was visualized by the migration of a bromophenol blue dye front – a constituent in the native loading buffer in which the proteins were prepared. A typical run would take 1.5 hours after which the separation was manually stopped. It was imperative that the run be stopped before the bromophenol blue dye front entered the buffer strip. If this happened, there would be no way to accurately make Rf determinations.

Gel staining

Gels were stained with Coomassie Blue for 1h and then destained in 30% methanol, 10 % acetic acid. Care was taken to not destain too long, as this would result in fading of the bromophenol blue dye front.

Calculations

A picture of the gel was taken and imported into Microsoft Paint 2007. Since it is known that the start of the resolving gel is 8% polyacrylamide and the end is 25%, the gel was carefully divided into 0.5 % sections by resizing the picture to 1700 pixels in length, and placing a line through the gel every 50 pixels.

Retardation factor (Rf) was determined at three gel concentrations by dividing the distance the protein migrated by the distance the Bromophenol Blue dye front migrated. The distance was measured in units of pixels in Microsoft Paint 2007. It was important to pay attention to the fact that Bromophenol Blue migration changed with respect to gel percentage – the higher the gel concentration the lower the migration distance. $\Delta Rf/(\Delta Gel\%)\Delta Rf/(\Delta Gel\%)$ was calculated for each protein, giving a slope characteristic of each molecular weight. The slopes were then plotted against Log (MW) to create a standard curve that was used to solve for an unknown MW.

RESULTS AND DISCUSSION

The nature of a transverse pore gradient polyacrylamide gel causes the loaded protein on the gel to appear unusual. Unlike the non-continuous protein bands seen in a normal pore gradient, a transverse pore gradient exhibits discrete bands with lateral continuity between adjacent wells. This is because each well is a representation of different polyacrylamide gel percentages and therefore has different effects on the migration patterns of the proteins. At the far left of Fig. 1 which is close to 25% polyacrylamide, the protein migration distances are very low; however, as one continues to follow the gradient into lower polyacrylamide gel percentages, the migration distances increase.

In Figure 1, it is also important to note that Bromophenol Blue migration also changed with respect to gel percentage – the higher the gel concentration the lower the migration distance. Carefully noting the variation will ensure accurate calculation of Rf values throughout the varying concentrations of the gel.

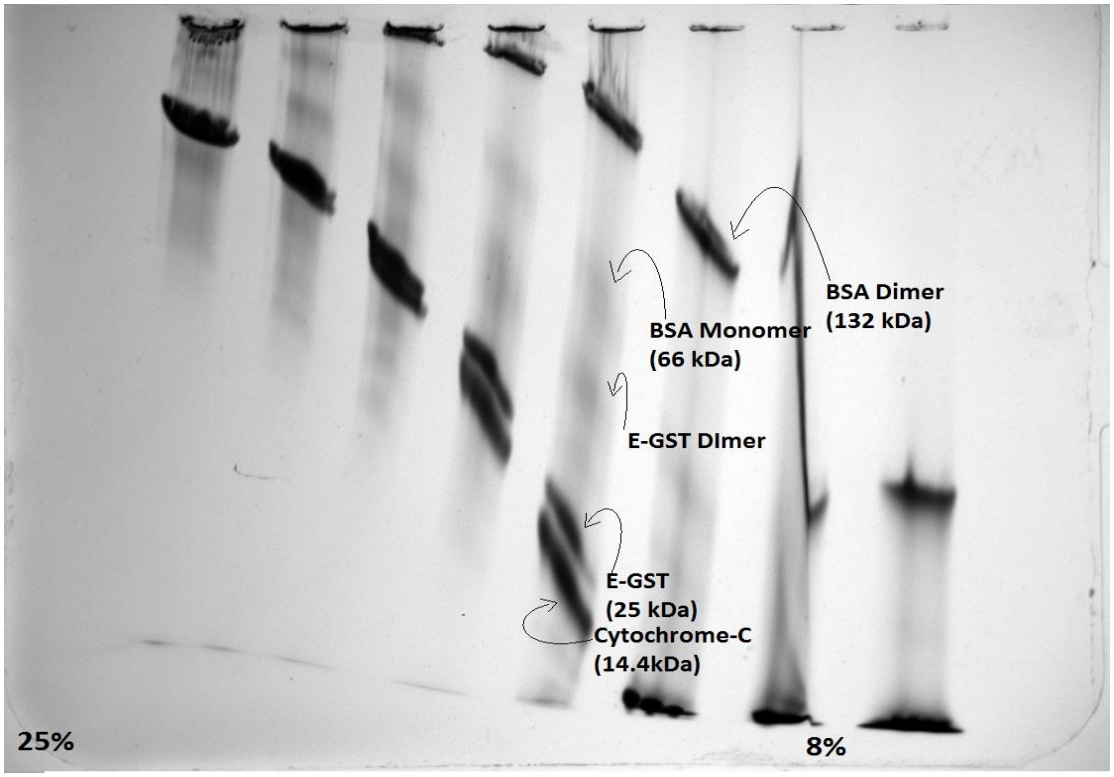

Figure 1: Transverse Pore Gradient (8-25%) Native PAGE of Cytochrome-C, E-GST, E-GST Dimer, BSA, and BSA Dimer

The concentration of each protein loaded onto the gel was 10ug/uL, a rather high concentration compared to many biochemical techniques. There are two significant reasons as to why such high protein concentration was used. The first reason has to do with the nature of protein migrating through an electric field strictly by its own native charge. Typically the native charge of a protein is rather low in magnitude, causing migration time to be very slow. A slow migration gives more opportunity for protein diffusion to occur into the surrounding gel matrix, which results in protein bands fading rather significantly. To counter this phenomenon, we added enough protein to the gel to ensure that there is a significant amount of protein left in the band after diffusion. This phenomenon occurs in standard Ferguson plots as well, and is therefore not unique to our method. Unique to our method is the need to identify a starting point on the gel where band migration begins. Since the gel is turned 90 degrees when run, we do not have the interface between the stacking and resolving gel as a reference, so instead we rely on a natural occurrence in proteins at very high concentrations. Proteins at very high concentrations will tend to aggregate, and therefore become far too large to enter the pores of the polyacrylamide gel. These protein aggregates are located at the exact spot on the gel where the comb was introduced, and therefore becomes an excellent reference point for the start of protein migration.

The voltage pattern used for running the gel was determined empirically. Ultimately the voltage described in the method section leads to optimal separation. This voltage pattern also happens to be the manufacturer's preprogrammed method for running a 12.5% Native Gel on the PhastSystem. In the case that the instrument was not preprogrammed, one should refer to the PhastSystem's owner manual for programming information and instruction.

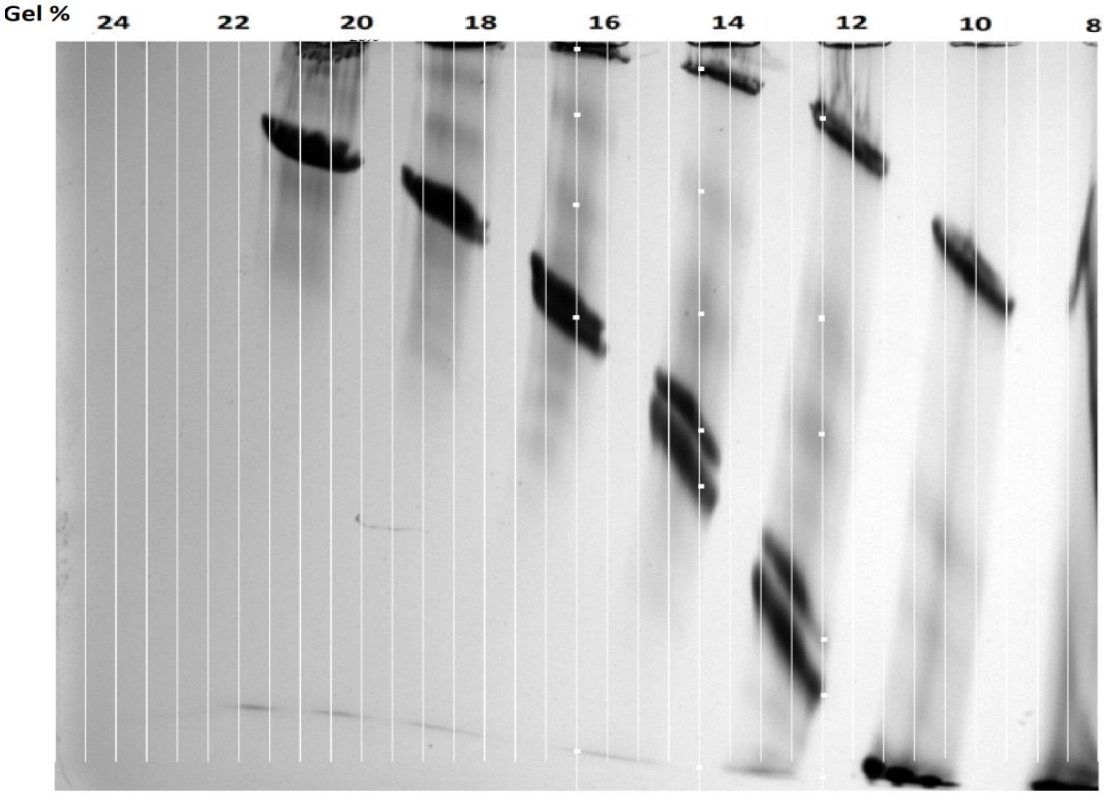

Figure 2: Processing of Transverse Pore Gradient (8-25%) Native PAGE in Microsoft Paint 2007

The overall pattern created on the gel is a series of curves that are logarithmic in nature each representing one protein or protein complex. Three points from a region of reasonably high linearity (from ~12.25% to ~16.25%) were used to calculate $\Delta Rf/(\Delta Gel\%)\Delta Rf/(\Delta Gel\%)$ for each band (Figure 2). A calibration curve of the log (MW) vs. $\Delta Rf/(\Delta Gel\%)$ $\Delta Rf/(\Delta Gel\%)$ (Figure 3) allows the molecular weight determination of E-GST Dimer. The calibration curve generated using this technique has an R^2-value of 0.97, indicating that the regression line accurately approximates the real data points. The regression equation (Figure 3) predicts the molecular weight of E-GST dimer to be 45.1 kDa, which is a 9% deviation from the expected value of 50 kDa.

A deviation of 9% seems slightly high when the regression line's R^2-value is 0.97, but can be explained by a disproportionate change in two protein characteristics upon homodimerization. When a protein forms a homodimer its molecular weight doubles but its surface area does not. The interface between the two subunits reduces surface area. So if MW increases by 100%, one can only be certain that surface area will increase less than 100%. Since a protein's surface interacts with the gel matrix, less surface area would cause less interaction and allow the protein to move further through the matrix. This disproportionate change in mobility with respect to MW could in part explain the 9% deviation observed.

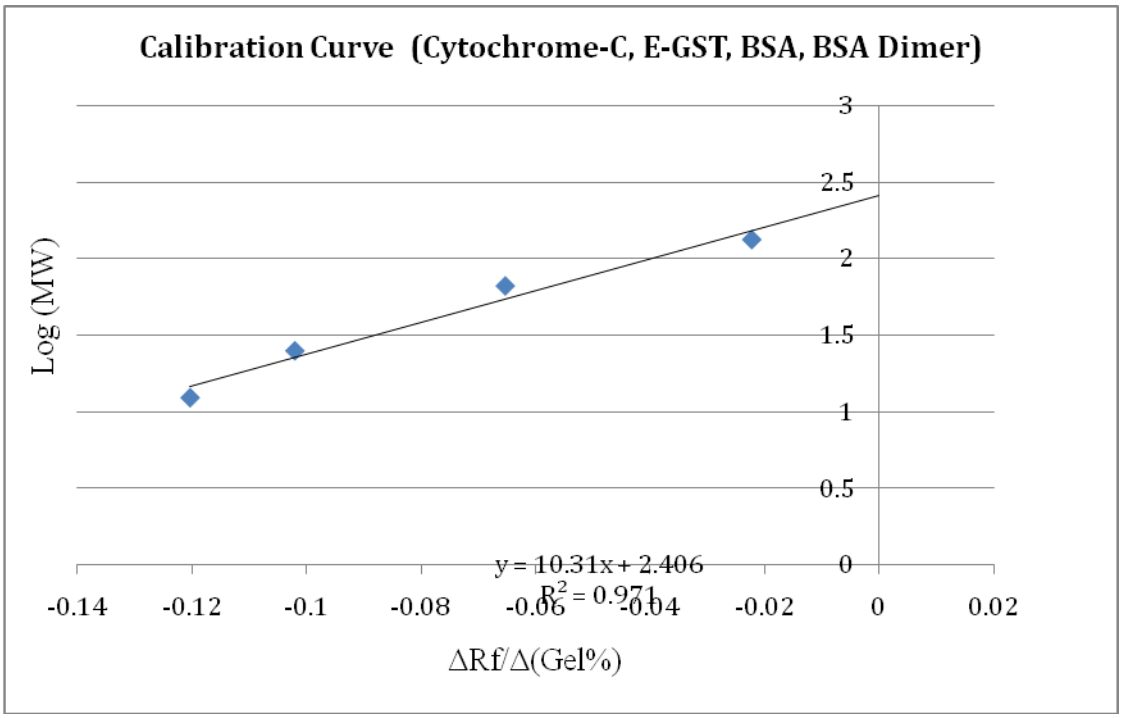

Figure 3: Plot of Log (MW) vs. ΔRf/Δ (Gel%) for Cytochrome-C, E-GST, BSA, and BSA Dimer

There are many advantages to native molecular weight determination using a transverse pore gradient with the PhastSystem in comparison to creating a Ferguson plot using standard methods. The most obvious is that a standard Ferguson plot requires running 3 or more gels while the method described herein requires the use of only one gel. The reason at least 3 gels must be run is that it is impossible to make a calibration curve with only two points. The one gel that is used in our method gives us the informational equivalent of running approximately 10-12 individual gels using the standard Ferguson method. Running the equivalent of 10-12 gels at one time allows for quick and easy identification of the gel percentage at which highest linearity occurs for all proteins, giving this method a higher accuracy compared to running individual gels. Furthermore, finding migration distances of proteins in many different acrylamide percentages on one gel reduces the innate experimental error due to small variations between gels. Multiple gel runs would certainly add new variables to each gel. While the variation may be insignificant between 3 gels, for example, it would have an additive effect and very likely become significant if one were to run 10-12 gels. Such variables include small variations in gel run time buffering capacity of the running buffer after multiple uses. Running one transverse pore gradient gel would also eliminate many potential issues in reproducibility of data for the same reasons.

In terms of economical benefits, this method is superior to the standard Ferguson plot in many ways. One transverse pore gradient gel takes 1.5 hours of run time, while the informational equivalent of about 10-12 individual gels of different percentages could take as long as 15-18 hours. This cuts into a significant amount of time in which a researcher can be doing something more productive, making our method superior in efficiency. Furthermore, money is saved when one gel is used rather than 10-12.

While there are many advantages in employing this technique, there are a number of drawbacks as well. The first drawback is the time it takes to analyze the gel. After staining

and destaining the gel, a picture must be taken and processed using Microsoft Paint 2007. It is imperative that the gel picture taken has the edge of the gel exactly parallel with the edge of the paper; this will make it possible to use the edges as reliable percentage references. The distance between the end of the gel opposite the tab (25%) and the stacking gel (8%) must be divided carefully into vertical sections each representing a 1% change in polyacrylamide concentration. This is achieved by opening the Ruler function in paint, then resizing the picture in accordance with the number of equal divisions that must be made. In this case, the picture was sized to 1700 pixels and marked every 50 pixels for 33 lines in total. Since the edges also count as percentage indicators there were 35 lines total, allowing division into 0.5% increments (Figure 2). The Rf values can then be measured in pixels, since the unit of measurement is arbitrary to Rf. Each migration distance was divided by bromophenol blue migration distance for the corresponding concentration to obtain Rf, and $\Delta Rf/(\Delta Gel\%)\Delta Rf/(\Delta Gel\%)$ is calculated. A standard curve of Log (MW) vs. $\Delta Rf/(\Delta Gel\%)\Delta Rf/(\Delta Gel\%)$ was plotted for Cytochrome-C, E-GST, BSA and BSA Dimer has an R^2 –Value of 0.9845, indicating the prediction it makes on the molecular weight of E-GST dimer will be accurate (Figure 3). While the calculations described are common to both the standard Ferguson plot and our transverse pore method, the measurement and migration linearity analysis described is an additional step unique to our method. Since this unique step does take time and care, it can be seen as a slight drawback when using our technique.

The most significant drawback to this technique is that its availability for use is predicated on the researcher already having a PhastSystem in their lab. The purchase of the PhastSystem would be a significant expense. However for labs that must deal with a lot of MW determinations of protein complexes or native proteins, the benefits of using this technique could conceivably outweigh the costs of purchasing the PhastSystem. Additionally, purchase of the PhastGel System has more uses than just our technique; they include SDS-PAGE, Native-PAGE, and Isoelectric Focusing.

In summation, the benefits of this technique include saving significant amounts of time and money; and generating highly accurate and reproducible MW determinations of native proteins and protein complexes. The drawbacks of this technique include the need for careful gel analysis prior to calculation and the need for a PhastGel system. The drawback of time used for gel analysis however is outweighed by the benefit of time saved in avoiding the running of 3 or more individual gels of different percentages. This technique is a more efficient, more cost effective, and more reproducible alternative to the standard Ferguson plot.

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REFERENCES

Andrews, A. T. (1981). Electrophoresis: Theory, techniques, and biochemical and clinical applications. Oxford University Press, New York City, New York.

Buzas, Z., Wheeler, D. L., Garner, M. M., Tiezt, D., & Chrambach, A. (1994). Transverse pore gradient gel electrophoresis using the PhastSystem. *Electrophoresis, 15,* 1028-1031.

Ferguson, K. A. (1964). Starch-Gel Electrophoresis--Application to the Classification of Pituitary Proteins and Polypeptides. *Metabolism 13*, 985-1002.

Hames, B. D., & Rickwood, D. (1998). Gel electrophoresis of proteins: a practical approach. Oxford University Press, New York City, New York.

Hendrick, J. K., & A. L. Smith (1968). Size and charge isomer separation and estimation of molecular weights of proteins by disc gel electrophoresis. *Archives of Biochemistry and Biophysics, 126,* 155-164.

Hou, S. (2018). Photo-thermally enhanced temperature gradient gel electrophoresis for DNA separation A Dissertation Proposal Presented to The Department of Chemical Engineering, Northeastern University, Boston, MA, USA

Laemmli, U. K. (1970). Cleavage of Structural Proteins during the Assembly of the Head of Bacteriophage T4. *Nature 227,* 690-685.

Rematal, C., & Babul, J. (1988). Determination of the molecular weight of proteins by electrophoresis in slab gels with a transverse pore gradient of crosslinked polyacrylamide in the absence of denaturing agents. *Annals of Biochemistry, 175,* 554-547.

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