Molecular Weight Determination by SDS-PAGE

Introduction

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) is a reliable method for determining the molecular weight (MW) of an unknown protein. The first step in MW determination of a protein is to separate the protein sample on the same gel with a set of MW standards. Next, a graph of log MW vs. relative migration distance (R_f) is plotted, based on the values obtained for the bands in the MW standard. The MW of the unknown protein band is then calculated by interpolation using this graph. The key to determining MW accurately is selecting separation conditions that will produce a linear relationship between log MW and migration within the likely MW range of the unknown protein.

SDS-PAGE Conditions for MW Determination

To ensure accurate MW determination, both the protein standards and the unknown protein must be electrophoresed on the same gel under identical separation conditions. It is also important to generate multiple data points (at least three gels) so that the estimated MW has statistical significance. The sample buffer used to solubilize the proteins should contain reducing agents (dithiothreitol or β-mercaptoethanol) to break disulfide bonds, which minimizes the effect of secondary structure on migration. In addition, a strong ionic detergent such as SDS is a required component of the sample buffer. SDS provides two functions: It denatures secondary, tertiary, and quaternary structures by binding to hydrophobic protein regions, and its binding confers a net negative charge on the proteins, which also results in a constant charge-to-mass ratio. The proteins are then separated through a gel in an electrical field according to their mass. However, other factors may also influence protein separation. These factors are discussed briefly under Limitations.

Analysis of Electrophoresed Proteins

An example of the approach is shown in Figure 1 using Green Fluorescent Protein (GFP) as a hypothetical example of an unknown protein. A dilution series of an *E. coli* lysate spiked with GFP and Precision Plus Protein™ unstained standards was electrophoresed in different lanes of a Criterion™ 4–20% SDS-PAGE gel, then stained with Bio-Safe™ Coomassie stain and destained in distilled water to visualize the protein bands. The gel was then analyzed to obtain the R_f values for each band. The R_f is defined as the migration distance of the protein through the gel divided by the migration distance of the dye front. The distance should be measured from the top of the resolving gel to the band of interest, as illustrated on the gel.

A plot of log MW versus R_f (Figure 2) was generated from the bands in the gel shown in Figure 1 to determine the MW of the unknown protein. The simplest method for this is to base the MW determination on a standard curve. If the curve is nearly linear, it can be described by the formula y = mx + b, where y is the log MW, m is the slope, x is the R_f, and b is the y-intercept, as shown in Figure 2.

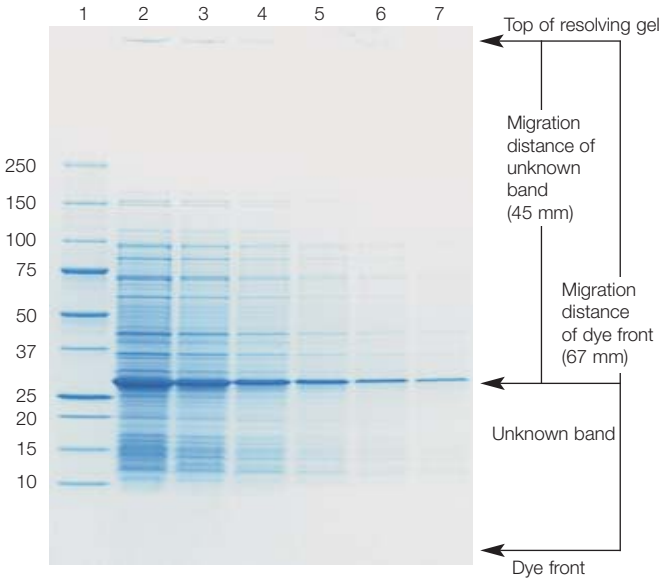

Fig. 1. Example showing approach for MW determination of an unknown protein. Lane 1, 10 µl of Precision Plus Protein unstained standards; lanes 2–7, a dilution series of an E. coli lysate containing a hypothetically unknown protein (GFP). Proteins were separated by SDS-PAGE in a Criterion 4–20% Tris-HCl gel and stained with Bio-Safe Coomassie stain. Gel is shown actual size. MW standards are in kD.

Alternatively, Bio-Rad's The Discovery Series™ Quantity One® 1-D analysis software, or software with similar capabilities, can be used to determine the R_f values. Quantity One is a tool for imaging and analyzing 1-D electrophoresis gels, dot blots, slot blots, and colony counts. For accurate MW determination, the unknown protein should be within the linear range of the standard curve, and the amount of the unknown protein (or its intensity after staining) should match the corresponding

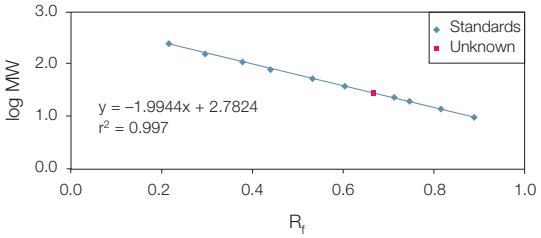

Fig. 2. Determining the MW of an unknown protein by SDS-PAGE. A standard curve of the log MW versus R_f was generated using the Precision Plus Protein standards from Figure 1. The strong linear relationship ($r^2 > 0.99$) between the proteins' MW and migration distance demonstrates exceptional reliability in predicting MW.

standard; in Figure 1, the unknown bands in lanes 5 and 6 are optimal. A gradient gel (for example, 4–20%) is generally used to determine the range where the unknown protein's MW falls, because the gradient allows proteins spanning a wide MW range to be examined. Single-percentage gels can then be used to further analyze the unknown protein. For a single-percentage gel, it is important to determine the MW range in which the relationship of log MW to migration distance of the standards is linear. The accuracy of the calculated MW depends on the linearity of the relationship, represented by the r^2 value. The closer the r^2 value is to 1.0, the better the fit of the data points to a line. If a set of protein standards does not generate a linear relationship, it is acceptable to remove the data points that lie beyond the linear range, as shown in Figure 3. In Figure 3B, the values for the five largest proteins were omitted from the calculations, increasing the r^2 value from 0.913 to 0.997. Most scientific calculators and software can generate the r^2 value and the equation of the best-fit line from the data points. A comparison of results obtained using a nonlinear and a linear curve is given in the Table.

Procedure

Figures 1 and 2 illustrate the procedure.

1. Run the standards and unknown on an SDS-PAGE gel.
2. Process the gel with the desired stain and then destain to visualize the protein bands.
3. Determine the R_f graphically or using Quantity One software (or equivalent).
4. Use a graphing program to plot the R_f versus log MW. From the program, generate the straight line equation y = mx + b, and solve for y to determine the MW of the unknown protein.

Determining R_f Graphically

- Use a ruler to measure the migration distance from the top of the resolving gel to each standard band and the dye front
- Calculate the R_f value of each band using the following equation:
  R_f = migration distance of the protein/ migration distance of the dye front

Determining R_f With Quantity One Software

- The R_f and r^2 values are determined automatically by the software (see Quantity One manual)

Example of Calculation

From Figure 1:

Migration distance of unknown protein: 45 mm

Migration distance of dye front: 67 mm

So R_f = 45 mm/67 mm = 0.67

From Figure 2:

$y = -1.9944x + 2.7824$

$x = R_f$ of unknown protein = 0.67

y = log MW

So $MW = 10^y = 10^{-1.9944(0.67) + 2.7824} = 28.1$ kD

The actual MW of GFP is 28.3 kD, so the accuracy of the observation using these standards was 99.2%, which is below the level of error inherent in making the measurements. Note that calculation based on the nonlinear curve (see Table) would not have been as accurate; if values had been calculated based on the entire set of standards shown in Figure 3, the predicted value for GFP would have been 37.0 kD.

Limitations

MW determination by SDS-PAGE is a dependable method. However, an unknown protein's MW should always be obtained by mass spectrometry if a more precise MW determination is needed. Mass spectrometry has a higher degree of accuracy because each amino acid of a protein is analyzed. Protein-to-protein variation can be minimized by denaturing samples, reducing proteins, normalizing the charge-to-mass ratio, and electrophoresing under set conditions. However, factors such as protein structure, posttranslational modifications, and amino acid composition are variables that are difficult or impossible to minimize and can affect the electrophoretic migration.

A few examples demonstrate the effect of these variables. Glycoproteins migrate unpredictably in SDS-PAGE (Hames 1998). The hydrophilic glycan moieties can obstruct the binding of SDS, and the decreased hydrophobic interaction between the protein and SDS result in an inconsistent charge-to-mass ratio. However, some evidence suggests that glycoproteins exhibit more normal protein migration in gradient gels. Acidic proteins (such as tropomyosin) also migrate abnormally on SDS-PAGE gels. The acidic residues may be repelled by the negatively charged SDS, leading to an unusual mass-to-charge ratio and migration. Highly basic proteins

Table. Comparison of results using nonlinear and linear ranges of standards to determine MW of an unknown protein. The protein was run on three Criterion 15% SDS-PAGE gels with Precision Plus Protein unstained standards to determine its MW. The accuracy of the MW determination is dependent on the linearity of the curve; curves used were those in Figure 3. The hypothetically unknown protein was GFP.

	Nonlinear Curve	Linear Curve
MW determination*	37.0 kD	29.3 kD
Accuracy of MW estimate	69.3%	96.5%

* Actual MW is 28.3 kD based on amino acid sequence.

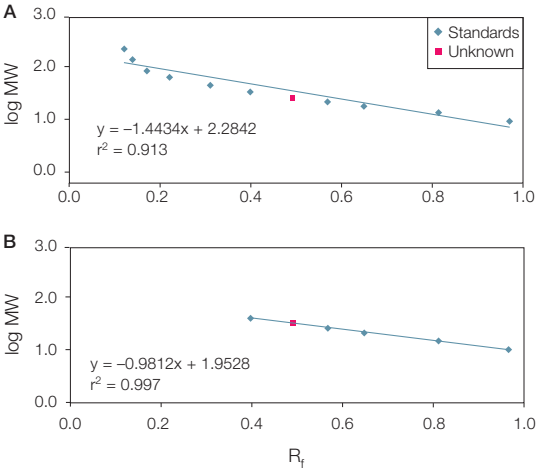

Fig. 3. Comparison of nonlinear and linear curves from a 15% Criterion SDS-PAGE gel. A, a plot of the log MW versus R_f generated using the entire protein standard range demonstrates a nonlinear curve, resulting in inaccurate MW determination. B, a curve generated from the proteins in the linear range (10–37 kD). The extreme points were removed, producing a linear curve and thus an accurate MW determination.

(for example, lysozyme, histones, and troponin I), which contain an abundance of positively charged amino acids, migrate more slowly in SDS-PAGE due to a reduced charge-to-mass ratio, resulting in a higher apparent MW. Proteins with high proline content or with other unusual amino acid sequences (for example, ventricular myosin light chain) show a decreased electrophoretic mobility as a result of kinks and structural rigidity caused by the primary sequence. These differences can contribute to an error of ±10% when using SDS-PAGE to determine the MW of a protein. Following SDS-PAGE analysis with mass spectrometry will produce a more accurate determination. Despite these limitations, SDS-PAGE is still a commonly used method for MW determination of a protein.

Reference

Hames BD (ed), Gel Electrophoresis of Proteins: A Practical Approach, 3rd edn, Oxford University Press, Oxford, New York (1998)

Ordering Information

Description

Criterion Tris-HCl Gels*

	12+2 Comb 45 µl Samples	18-Well Comb 30 µl Samples	26-Well Comb 15 µl Samples
5% Resolving Gel	345-0001	345-0002	345-0003
7.5% Resolving Gel	345-0005	345-0006	345-0007
10% Resolving Gel	345-0009	345-0010	345-0011
12.5% Resolving Gel	345-0014	345-0015	345-0016
15% Resolving Gel	345-0019	345-0020	345-0021
18% Resolving Gel	345-0023	345-0024	345-0025
4–15% Linear Gradient	345-0027	345-0028	345-0029
4–20% Linear Gradient	345-0032	345-0033	345-0034
8–16% Linear Gradient	345-0037	345-0038	345-0039
10–20% Linear Gradient	345-0042	345-0043	345-0044
10.5–14% Linear Gradient	345-9949	345-9950	345-9951

Catalog #	Description
Precision Plus Protein Standards and Conjugates	
161-0363	Precision Plus Protein Unstained Standards, 1 ml
161-0373	Precision Plus Protein All Blue Standards, 500 µl
161-0374	Precision Plus Protein Dual Color Standards, 500 µl
161-0375	Precision Plus Protein Kaleidoscope™ Standards, 500 µl
161-0380	Precision Protein™ StrepTactin-HRP Conjugate, 300 µl
161-0382	Precision Protein StrepTactin-AP Conjugate, 300 µl
Electrophoresis Reagents	
161-0737	Laemmli Sample Buffer, 30 ml
161-0772	10x Tris/Glycine/SDS, 5 L cube
161-0787	Bio-Safe Coomassie Stain, 5 L cube
Blotting Reagents	
170-8236	Opti-4CN™ Goat Anti-Rabbit Detection Kit
170-8237	Opti-4CN Goat Anti-Mouse Detection Kit
170-8239	Amplified Opti-4CN Goat Anti-Rabbit Detection Kit
170-8240	Amplified Opti-4CN Goat Anti-Mouse Detection Kit
170-6460	Immun-Blot® Goat Anti-Rabbit IgG (H + L)-AP Kit
170-6461	Immun-Blot Goat Anti-Mouse IgG (H + L)-AP Kit
170-6463	Immun-Blot Goat Anti-Rabbit IgG (H + L)-HRP Kit
170-6464	Immun-Blot Goat Anti-Mouse IgG (H + L)-HRP Kit
170-6432	AP Conjugate Substrate Kit
170-6431	HRP Conjugate Substrate Kit
161-0734	10x Tris/Glycine, 1 L
161-0771	10x Tris/Glycine, 5 L cube
170-6435	10x Tris-Buffered Saline, 1 L
161-0781	10% Tween 20, 1 L
Blotting Membranes and Filter Paper	
162-0112	Nitrocellulose Membrane, 0.2 µm, 30 cm x 3.5 m, 1 roll
162-0115	Nitrocellulose Membrane, 0.45 µm, 30 cm x 3.5 m, 1 roll
162-0177	Immun-Blot PVDF Membrane, 26 cm x 3.3 m, 1 roll
162-0184	Sequi-Blot™ PVDF Membrane, 26 cm x 3.3 m, 1 roll
170-3956	Trans-Blot™ Thick Filter Paper, 15 x 20 cm, 25 sheets

Catalog #	Description
Blotting Membrane/Filter Paper Sandwiches (7 x 8.5 cm)**	
162-0216	Sequi-Blot PVDF/Filter Paper Sandwiches, 7 x 8.5 cm, 20 pack
162-0217	Sequi-Blot PVDF/Filter Paper Sandwiches, 7 x 8.5 cm, 50 pack
162-0212	Nitrocellulose/Filter Paper Sandwiches, 0.2 µm, 7 x 8.5 cm, 20 pack
162-0213	Nitrocellulose/Filter Paper Sandwiches, 0.2 µm, 7 x 8.5 cm, 50 pack
162-0214	Nitrocellulose/Filter Paper Sandwiches, 0.45 µm, 7 x 8.5 cm, 20 pack
162-0215	Nitrocellulose/Filter Paper Sandwiches, 0.45 µm, 7 x 8.5 cm, 50 pack
Blotting Membrane/Filter Paper Sandwiches (8.5 x 13.5 cm)**	
162-0236	Sequi-Blot PVDF/Filter Paper Sandwiches, 8.5 x 13.5 cm, 20 pack
162-0237	Sequi-Blot PVDF/Filter Paper Sandwiches, 8.5 x 13.5 cm, 50 pack
162-0232	Nitrocellulose/Filter Paper Sandwiches, 0.2 µm, 8.5 x 13.5 cm, 20 pack
162-0233	Nitrocellulose/Filter Paper Sandwiches, 0.2 µm, 8.5 x 13.5 cm, 50 pack
162-0234	Nitrocellulose/Filter Paper Sandwiches, 0.45 µm, 8.5 x 13.5 cm, 20 pack
162-0235	Nitrocellulose/Filter Paper Sandwiches, 0.45 µm, 8.5 x 13.5 cm, 50 pack
Equipment	
165-6001	Criterion Cell, includes electrophoresis buffer tank, lid with cables, 3 sample loading guides (12+2 well, 18-well, 26-well), instructions
170-4070	Criterion Blotter With Plate Electrodes, includes cell assembled with plate electrodes, lid with cables, 2 Criterion gel holder cassettes, 1 package precut blot absorbent filter paper, 4 fiber pads, gel/blot assembly tray, roller, sealed ice block, instructions
170-4071	Criterion Blotter With Wire Electrodes, includes same as 170-4070 except cell assembled with wire electrodes

* All gels have a 4% stacking gel except 4–15% and 4–20%.

** Each sandwich consists of one membrane and 2 sheets of thick filter paper cut to fit Criterion (13.3 x 8.7 cm) or Ready Gel® precast gels (8.6 x 6.8 cm).

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