Using Precision Plus Protein™ Standards to Determine Molecular Weight

Introduction

Use of Standards to Determine Molecular Weight (MW) of Unknown Proteins

Protein standards are a convenient tool used with SDS-PAGE to determine the MW of unknown proteins. They consist of a mixture of proteins of known MW. In addition to their use in determining the MW of sample proteins, they are used to monitor electrophoresis runs and to determine transfer efficiency on western blots. Protein standards are composed of natural or recombinant proteins, which can be either unstained or prestained. The proteins in a standard are electrophoretically separated by size, and the resulting band pattern, called a protein ladder, is used to generate a standard curve that can be used to predict the size of unknown proteins. The standard curve is generated by plotting the log of the MW vs. relative migration distance (R_f value) of each standard band. The MW of the unknown protein can then be calculated by interpolation on the standard curve based on its R_f value. For a detailed description of the methods and calculations used to determine the MW of a sample protein using SDS-PAGE, request bulletin 3133.

Factors Influencing Protein Migration

The electrophoretic migration of a protein depends on three factors: size, structure, and electrical charge. To eliminate structure and charge as factors, protein standards are usually suspended in a Laemmli sample buffer. A traditional Laemmli system consists of five components:

- Buffer: Tris, pH 6.8 (62.5 mM)
- Density agent: glycerol (10–30%)
- Denaturing agent: sodium dodecyl sulfate (SDS, 2%)
- Reducing agent: dithiothreitol (DTT, ≥50 mM)
- Tracking dye: Bromophenol Blue (0.1%)

Laemmli buffer helps stabilize proteins and ensure consistent migration. Glycerol increases the density of the protein solution so it settles in the well during sample loading. SDS is an anionic detergent added to minimize structure and charge as factors influencing protein migration. SDS coats proteins at a mass ratio of 1.4:1, eliminating most of a protein's complex structure. The SDS-coated protein will now have a net negative charge and will be strongly attracted to the anode (positive electrode) during SDS-PAGE. DTT is a reducing agent, also used to eliminate structure as a factor influencing protein migration. Reduction breaks disulfide bonds and exposes more of a protein's hydrophobic regions, allowing SDS to bind to these regions of the protein. Bromophenol Blue is used as a tracking dye to monitor the progress of an electrophoresis run; generally, for MW determination, the gel is run until the tracking dye nears the bottom of the gel. The distance migrated by the dye during the run is then used as the point of reference when calculating the R_f value of standards and sample proteins.

Although proteins suspended in a Laemmli buffer will have a net negative charge and a flexible rod shape during electrophoresis, other factors can still influence electrophoretic migration. For instance, posttranslational modifications of natural proteins, such as the addition of carbohydrate units, phosphorylation, and hydroxylation, alter both the mass and the mobility of a protein. In addition, the amino acid sequence affects protein migration by giving the protein a net charge. Proteins with a net positive charge run more slowly than proteins with a net negative charge. For instance, lysozyme, a lysine-rich protein with a net positive charge, migrates more slowly than most proteins of the same MW. Conversely, proteins with a net negative charge due to an abundance of glutamate or aspartate residues will migrate more quickly, resulting in a lower apparent MW. Because these factors influence migration in a gel, they will affect the apparent MW of both standards and unknown proteins. Even if the protein standards used to determine the MW of an unknown protein are very accurate, proteins with an unusual composition that do not migrate according to their actual MW make precise MW determination impossible. For these reasons, secondary methods of MW determination (for example, mass spectrometry) should be used for confirmation.

Factors Influencing the Usefulness of Protein Standards

Unstained protein standards cannot be visualized without the aid of stains such as Coomassie Blue, silver stain, and SYPRO Ruby. In contrast, prestained standards are covalently dyed, allowing visualization of the ladder during electrophoresis, and can be used to monitor the electrophoretic run. To precisely measure the R_f value of unknown and standard proteins, it is essential to have intense, sharp bands. Until recently, unstained standards have been the choice for MW determination due to their sharp bands, while prestained standards have been considered inadequate due to their diffuse, smeared bands and inconsistency in apparent MW. Most of these problems arise from the staining process. For instance, due to various posttranslational modifications, natural prestained standards may suffer from nonuniform stain uptake, resulting in diffuse bands. Furthermore, both natural and recombinant prestained standards can be overloaded with dye, producing broad, smeared bands. Prestained standards with broad, diffuse, or smeared bands are not as useful as unstained standards in MW determination.

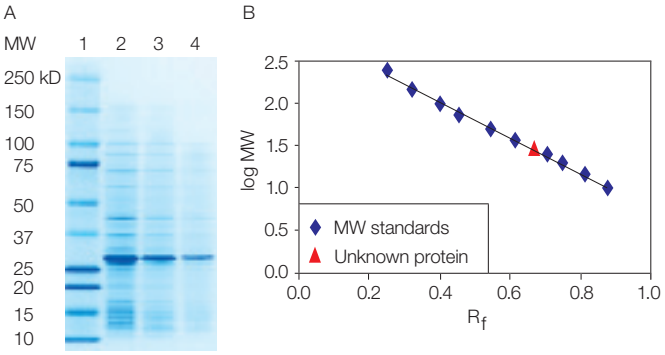

Fig. 1. Assessment of accuracy of MW determination using Precision Plus Protein standards. A, MW determination of an unknown protein using Precision Plus Protein Kaleidoscope prestained standards. The standards (lane 1) and a dilution series of an *E. coli* lysate (lanes 2–4) were electrophoresed on a Criterion 4–20% Tris-HCl precast gel and stained with Bio-Safe Coomassie Blue G-250 stain. Precision Plus Protein Kaleidoscope standards appear blue after Coomassie staining but retain the sharp, compact bands. B, the standards' r^2 value (0.99) and the MW of the unknown protein (28.6 kD) were calculated using Quantity One software. The true MW of the unknown protein (28.3 kD) was predicted by amino acid sequence analysis.

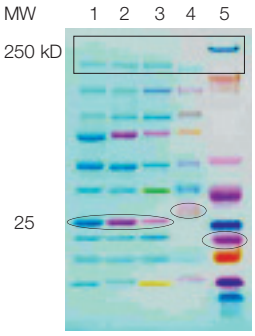

Fig. 2. Differences in the apparent MW of proteins from five protein standards. The uppermost protein in each standard (boxed), has a mass of 250 kD; the circled proteins have masses of approximately 25 kD. This gel shows the different migration patterns for each set of standards' 250 and 25 kD proteins. Precision Plus Protein all blue, dual color, and Kaleidoscope standards (lanes 1–3), Amersham's Rainbow (lane 4) standards, and Invitrogen's MultiMark (lane 5) standards were electrophoresed on a Criterion 4–20% Tris-HCl precast gel.

Precision Plus Protein Standards

Bio-Rad's recombinant Precision Plus Protein† standards overcome many of the traditional limitations of both unstained and prestained standards. All Precision Plus Protein standards have been engineered for predictable R_f values that correspond to their actual MW, and the MW of every lot is confirmed by mass spectrometry. These standards are available in unstained and prestained formats that migrate identically with an easily recognizable pattern. The prestained standards, which are available in a choice of three formats (all blue, dual color, and Kaleidoscope™ standards), are stained with a proprietary technology that ensures consistent, uniform staining, resulting in sharp bands.

Methods

Electrophoresis

Samples were suspended in Laemmli sample buffer and run on Criterion™ 4–20% Tris-HCl precast gels in a Criterion cell. Staining after electrophoresis, when necessary, was performed using Bio-Safe™ Coomassie Blue G-250 stain. Each standard was applied and electrophoresed at the manufacturer's recommended loading volume.

Accuracy and Linearity of Standard Curves

Standard curves were generated according to the method described in bulletin 3133. Quantity One® software was used to determine the r^2 values and to determine the MW of a hypothetically unknown protein.

To test the accuracy of Bio-Rad's Precision Plus Protein Kaleidoscope standards, the results of MW determination by electrophoresis were compared to the results of MW prediction based on amino acid sequence.

To compare the accuracy of several protein standards, Amersham's Rainbow, Invitrogen's MultiMark, and Bio-Rad's recombinant Precision Plus Protein standards were all run on the same gel so the results could be compared.

To compare the accuracy of natural protein standards against recombinant protein standards, Bio-Rad SDS-PAGE prestained standards and Precision Plus Protein Kaleidoscope standards were both used to determine the MW of the same unknown protein.

To determine the linearity of standard curves generated using Precision Plus Protein standards, the r^2 values for standard curves generated using the Precision Plus Protein family of standards were calculated. These values were compared to the r^2 value for Invitrogen's MultiMark MW protein standards.

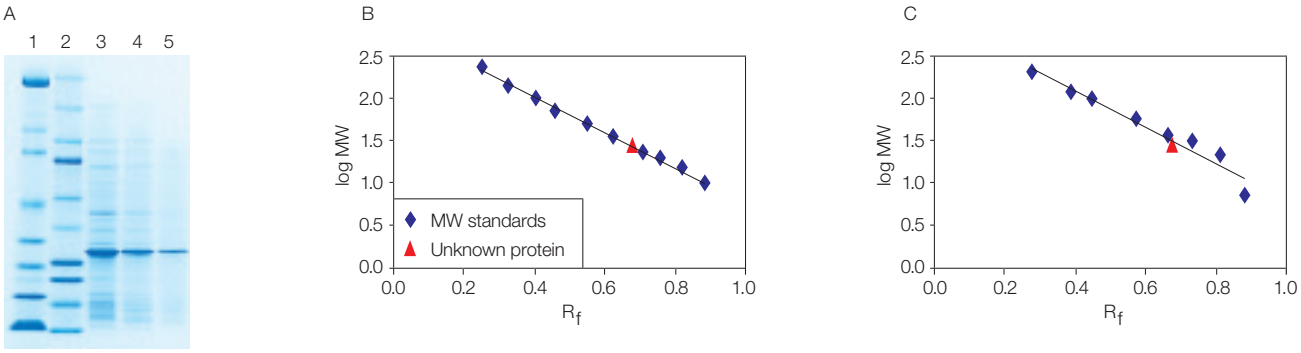

Fig. 3. Comparison of predicted MW of an unknown protein using two protein standards. A, Bio-Rad's SDS-PAGE prestained standards (lane 1), Precision Plus Protein Kaleidoscope (lane 2), and a dilution series of an *E. coli* lysate (lanes 3–5) were electrophoresed on a Criterion 4–20% Tris-HCl precast gel and stained with Bio-Safe Coomassie Blue G-250 stain. B, standard curve for Precision Plus Protein Kaleidoscope standards (r^2 = 0.997), giving a calculated MW of 28.6 kD. C, standard curve for Bio-Rad's SDS-PAGE prestained standards (r^2 = 0.950), giving a calculated MW of 32.1 kD. The true MW is 28.3 kD. The r^2 values for each set of standards and the predicted MW of the unknown protein were determined using Quantity One software.

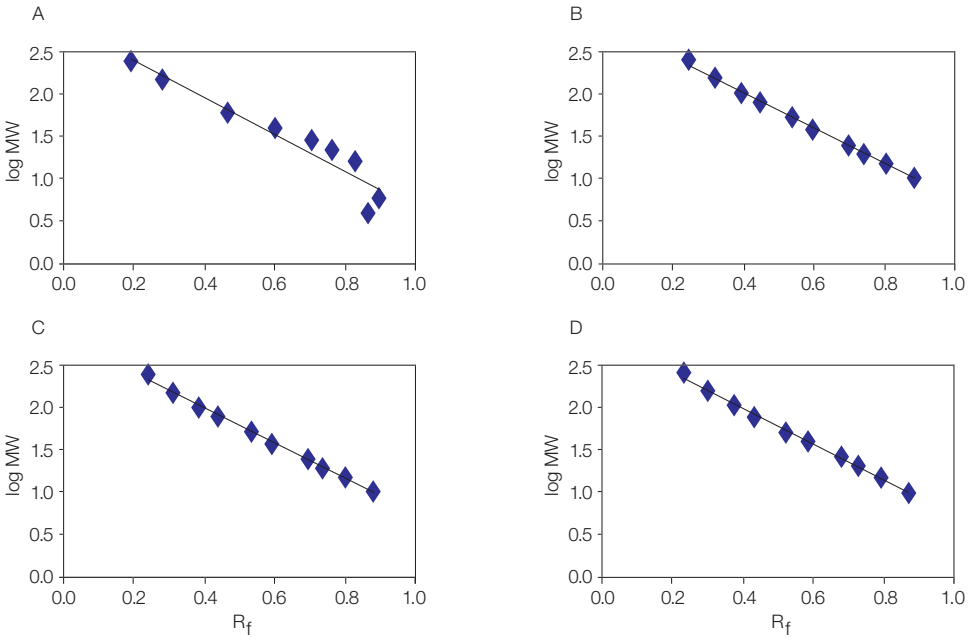

Fig. 4. Comparison of r^2 values for prestained standards. Invitrogen's MultiMark (A) and Bio-Rad's Precision Plus Protein dual color (B), Kaleidoscope (C), and all blue (D) standards. Each set of standards was electrophoresed on a Criterion 4–20% Tris-HCl precast gel, and the r^2 values (0.913, 0.996, 0.997, and 0.997, respectively) were determined using Quantity One software.

Results

Figure 1 illustrates the accuracy with which Precision Plus Protein Kaleidoscope standards were used to determine the MW of an unknown protein. Precision Plus Protein Kaleidoscope standards combine the accuracy of a recombinant unstained standard with the convenience of a multicolored, prestained standard. Bio-Rad's proprietary staining technology does not cause smeared or broadened bands, or substantially alter apparent MW.

As shown in Figure 2, proteins of the same mass from several protein standards did not migrate identically. These apparent differences in the MWs of proteins of the same mass resulted from varying staining chemistry and protein composition among standards.

As shown in Figure 3, when several protein standards were all run on the same gel, the calculated MW of the unknown protein differed by more than 10% between standards.

Figure 4 illustrates the exceptional linearity of all Precision Plus Protein standards.

Discussion

When choosing a protein standard, the following are important factors to consider:

- MW range of proteins of interest in the sample mixture
- Need for consistency in electrophoretic migration
- Intensity and sharpness of staining

Since different standards utilize different staining chemistries and contain proteins with different amino acid composition, the slopes and r^2 values for two standards will not be identical. Therefore, using more than one standard to determine the MW of an unknown protein will lead to different conclusions. Consequently, once the appropriate MW standard is chosen, the same standard should be used throughout a project. For example, when Bio-Rad's SDS-PAGE prestained and Precision Plus Protein Kaleidoscope standards were both used to determine the MW of the same unknown protein, the calculated MW of the unknown differed by more than 10% (Figure 3). To avoid such inconsistent results, once an unknown protein has been calibrated to a particular protein standard, only that standard should be used for MW determination.

Recombinant Precision Plus Protein standards contain amino acid sequences that ensure predictable migration, and each lot is tested by mass spectrometry, making them the most reliable protein MW standards on the market. The proprietary technology used to stain Precision Plus Protein Kaleidoscope standards makes them the prestained standard most suitable for MW determination. These standards have an easily recognizable migration pattern, allowing data comparison across different gel types, blots, and images. Since each protein is related, r^2 values are very high for standard curves generated by every member of the Precision Plus Protein family.

Ordering Information

Description	12+2 Comb 45 µl Samples	18-Well Comb 30 µl Samples	26-Well Comb 15 µl Samples
Criterion Tris-HCl Gels*			
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, 1.0 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 Electrophoresis Buffer, 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-Alkaline Phosphatase Kit
170-6461	Immun-Blot Goat Anti-Mouse-Alkaline Phosphatase Kit
170-6463	Immun-Blot Goat Anti-Rabbit-Horseradish Peroxidase Kit
170-6464	Immun-Blot Goat Anti-Mouse-Horseradish Peroxidase Kit
170-6432	Alkaline Phosphatase Conjugate Substrate Kit
170-6431	Horseradish Peroxidase Conjugate Substrate Kit
161-0734	10x Tris/Glycine Transfer Buffer, 5 L cube
170-6435	10x Tris Buffered Saline (TBS), 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 roll
162-0115	Nitrocellulose Membrane, 0.45 µm, 30 cm x 3.5 m roll
162-0177	Immun-Blot PVDF Membrane, 26 cm x 3.3 m roll
162-0184	Sequi-Blot™ PVDF Membrane, 26 cm x 3.3 m roll
170-3956	Thick Blot Absorbent Filter Paper, 15 x 20 cm, 25 sheets

Conclusions

Determining MW of sample proteins is a basic goal of many studies. Although the gold standard for determining the MW of a protein is mass spectrometry, this technique involves time-consuming and costly steps. SDS-PAGE using Precision Plus Protein standards is the most accurate, cost-effective alternative to mass spectrometry available for protein MW determination.

Reference

Molecular weight determination by SDS-PAGE, Bio-Rad bulletin 3133 (2004)

Catalog #	Description
Blotting Membrane/Filter Paper Sandwiches**	
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
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
Image Analysis Systems and Software	
170-3742	Polaroid Standard Documentation System, 120 V, includes mini-transilluminator, DS-34 camera, standard electrophoresis hood, Deep Yellow DS-34 camera filter, 1 pack film
170-8060	Gel Doc™ EQ System, PC, includes darkroom, UV transilluminator, epi-white illumination, camera, PCI digitizing card, cables, Quantity One software, instructions
170-8061	Gel Doc EQ System, Mac, includes darkroom, UV transilluminator, epi-white illumination, camera, PCI digitizing card, cables, USB-to-serial converter, Quantity One software, instructions
170-8030	VersaDoc™ Model 3000 Imaging System, PC, 100/240 V, includes a 50 mm and 20–40 mm zoom lens, sample tray, cleaning kit, Quantity One software, instructions
170-8031	VersaDoc Model 3000 Imaging System, Mac, 100/240 V
170-9600	The Discovery Series™ Quantity One 1-D Analysis Software

* 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).

Information in this tech note was current as of the date of writing (2004) and not necessarily the date this version (Rev A, 2004) was published.

BIO-RAD

Bio-Rad Laboratories, Inc.

Life Science Group

Web site *www.bio-rad.com* ***USA*** *800 424 6723* ***Australia*** *61 2 9914 2800* ***Austria*** *01 877 89 01* ***Belgium*** *09 385 55 11* ***Brazil*** *55 31 3689 6600* ***Canada*** *905 364 3435* ***China*** *86 20 8732 2339* ***Czech Republic*** *420 241 430 532* ***Denmark*** *44 52 10 00* ***Finland*** *09 804 22 00* ***France*** *01 47 95 69 65* ***Germany*** *089 31 884 0* ***Greece*** *30 210 777 4396* ***Hong Kong*** *852 2789 3300* ***Hungary*** *36 1 459 6100* ***India*** *91 124 4029300* ***Israel*** *03 963 6050* ***Italy*** *39 02 216091* ***Japan*** *03 6361 7000* ***Korea*** *82 2 3473 4460* ***Mexico*** *52 555 488 7670* ***The Netherlands*** *0318 540666* ***New Zealand*** *0508 805 500* ***Norway*** *23 38 41 30* ***Poland*** *48 22 331 99 99* ***Portugal*** *351 21 472 7700* ***Russia*** *7 495 721 14 04* ***Singapore*** *65 6415 3188* ***South Africa*** *27 861 246 723* ***Spain*** *34 91 590 5200* ***Sweden*** *08 555 12700* ***Switzerland*** *061 717 95 55* ***Taiwan*** *886 2 2578 7189* ***United Kingdom*** *020 8328 2000*