Comparison of Protein Quantitation Methods Using the Experion™ Automated Electrophoresis System

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Introduction

Protein quantitation is a routine analysis procedure required for drug discovery, process development, product manufacturing, and quality control in the pharmaceutical and biopharmaceutical industries. The Experion automated electrophoresis system integrates protein quantitation into a single process in which protein separation, staining, band detection, and quantitation are automatically executed with little user intervention. Compared to conventional SDS-PAGE methods that use gel image analysis for quantitation, the automatic protein quantitation performed by Experion software significantly reduces the labor and time spent in data analysis. However, to use the Experion system as a quantitative tool for protein analysis, it is essential to fully understand and appropriately utilize the protein quantitation techniques provided in Experion software.

Experion software offers two different types of protein quantitation methods: percentage determination and concentration determination (Table 1). Percentage determination measures the percentage of each protein in a protein mixture and is commonly used for determining protein content, protein purity, and protein stability, and for checking for mutations. Concentration determination provides the amount of the protein(s) in a protein mixture rather than just a percentage of the total. There are several different ways that concentration determination can be performed, and these methods provide more or less precision depending on the type of internal standard used and the extent to which a calibration curve is used. The ability of the Experion system to provide these different methods of quantitation offers the user the flexibility to customize their quantitation experiments to meet their needs for both throughput and accuracy.

This article describes the percentage determination and concentration determination methods used by Experion software, the ways in which these methods are used to achieve different levels of accuracy in protein quantitation, and the considerations that must be made in selecting one method over another.

Percentage Determination

Percentage determination is a simple, straightforward method for applications not requiring determination of protein concentrations. Experion software automatically performs percentage determination for all identified proteins in a sample, expressing the amount of each protein as a percentage of the total protein in the sample. The method is highly reproducible, and does not require any internal standards, additional calibrant samples, or manipulations in Experion software. Percentage determination is best suited to routine comparisons of samples with the same or similar protein composition.

Table 1. Methods used by Experion software for protein quantitation.

		Calibration Method and Internal Standard (IStd) Used			
		Single-Point Calibration		Calibration Curve	
Quantitation Method*	Output	Upper Marker IStd	User-Defined IStd	Upper Marker IStd	User-Defined IStd
Percentage Determination	% Total	No internal standard required			
Concentration Determination					
Relative quantitation	ng/µl	•	—	—	—
		—	•	—	—
Absolute quantitation	ng/µl	—	—	•	—
		—	—	—	•

* Accuracy: absolute concentration determination > relative concentration determination. Accuracy for percentage determination is protein dependent.

Experion software bases all protein quantitation measurements on the time-corrected peak area (corrected area) of each peak identified in an electropherogram (Figure 1). The corrected area of a peak is proportional to the amount of the protein it represents in a mixture. To determine the percentage of total protein represented by each protein, Experion software determines the sum of the corrected areas of all identified peaks in an electropherogram (total area) and then reports the percentage of that sum that is represented by each peak (% total). Experion software automatically calculates a % total value for each protein and lists the results in the result table. The software also allows exclusion of one or more peaks from the % total calculation.

Unlike the concentration determination methods described below, percentage determination provides reproducible results without requiring the use of an internal standard. The internal standard is used to reduce the effects of experimental variations associated with a protein assay, variations that may adversely affect reproducibility. Since each protein in a sample is subjected to the same variations, relative protein abundance within the sample (measured by % total) will not be significantly affected.

Experiments have shown average coefficients of variation (CVs) of 5% for intrachip reproducibility (data not shown) and less than 8% interchip reproducibility (Table 2).

Table 2. Analysis of interchip reproducibility of percentage determination. Experiments were performed using eight different proteins from the Experion Pro260 ladder. Each protein was measured on two different instruments using a total of seven different chips on three successive testing days.

Protein Size (kD)	Number of Samples	Mean % Total	Standard Deviation	%CV
10	69	17.6	1.2	6.7
20	69	15.4	0.4	2.8
25	69	14.7	0.9	6.4
37	69	11.47	0.6	5.0
50	69	11.87	0.4	3.5
75	69	12.0	0.0	3.3
100	69	10.5	0.6	5.6
150	69	6.6	0.5	7.7

The accuracy of the % total method will depend on the dye-binding efficiency of each component in the protein mixture. As with other dye-based assays (Bio-Rad Laboratories 2003, Bradford 1976, Lowry et al. 1951) or other methods of quantitation using SDS-PAGE, differences in the amino acid sequences or structures of proteins result in their unique interaction with the Experion Pro260 dye, which in turn affects band intensity. As a result, the % total value may not always reflect the actual percentage in mass for each protein in a mixture. If the component is well-characterized, however, this method of quantitation can serve as an efficient method for processes requiring routine monitoring of protein samples with the same or similar protein compositions. In manufacturing processes where the Experion Pro260 assay is used to regularly monitor protein components, this quantitation method can be used to quickly and easily monitor the quality of products from time to time or from batch to batch (especially when the products are at the early stages of development) and to ensure that incoming materials meet specifications.

To maintain quality control in such product control processes, however, it is important to develop a standard sample against which the protein products and different analyses can be compared over time. This standard sample should contain the same components as the products being evaluated and should be constructed so that the peak area percentages of two or three proteins of interest (target proteins) match a set of predetermined acceptance criteria. The standard and product samples are then analyzed on the same chip. Quality control for the assay is achieved by comparing the peak area percentages of the target proteins in the standard to the acceptance criteria, and quality control for the products is achieved by comparing the peak percentages of the proteins of interest in the standard and product samples. Since the Experion system can analyze up to 10 protein samples in about 30 minutes, large numbers of products can be checked quickly and easily. If the Experion system is used to monitor a protein component(s) in an ongoing process, such as protein purification, users should perform simple method development to understand how the monitored protein component(s) interacts with the dye and what effect this component has on the protein percentage profiles in the sample throughout the process. If the user is able to characterize the key component(s) to be monitored, the peak area percentage quantitation can also be an easy, fast, and routine method for many ongoing protein processes.

Concentration Determination

Concentration determination calculates the protein amount(s) using one of two quantitation methods: relative (also known as estimated) concentration or absolute concentration determination. Relative concentration determination uses a single-point calibration against an internal standard to determine the concentrations of all identified proteins in a sample, while absolute concentration determination uses a standard curve generated using multiple concentrations of a purified protein. Absolute concentration is commonly used to more accurately measure the concentration of a specific protein.

Internal Standards

An internal standard is subjected to the same variations as the target protein(s), so the inclusion of an internal standard helps offset the effects of experimental variations that can negatively impact reproducibility and accuracy. Experion software uses the ratio of the corrected area of each protein to that of the internal standard, a ratio that is independent of bias, to calculate protein concentrations.

The default internal standard for both methods of concentration determination is the 260 kD upper marker (UM), which is included in the Experion Pro260 sample buffer and so is a component of each sample (Figure 1).

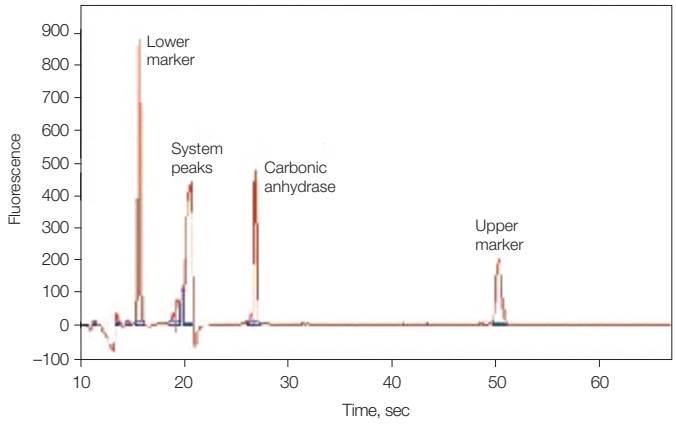

Fig. 1. Experion electropherogram. The positions of the upper marker (UM) and of a protein peak carbonic anhydrase are indicated. The UM is the default internal standard; however, carbonic anhydrase could be used as an internal standard.

Use of the UM generally provides accurate, reproducible quantitation; however, for increased accuracy in cases where a protein of interest might display different staining characteristics from the UM, Experion software allows use of a user-defined protein added at a known concentration. In many cases, this latter approach may provide more accurate results, provided that the standard:

- Is similar to the sample protein(s) in chemical structure and dye-binding efficiency
- Is not a natural component of the sample
- Behaves in a similar manner to the sample protein(s) during the sample preparation process
- Behaves in a similar manner to the sample protein(s) during separation and detection
- Separates as a distinct peak and does not interfere with the baseline resolution of the protein sample(s), the system peak, or the upper and lower markers
- Remains stable throughout separation and analysis
- Is added to each sample prior to sample preparation (so that any losses affecting the target proteins will similarly affect the internal standard) at the same concentration across the chip, preferably in an amount that is similar to that of the target protein in the sample

Relative Concentration Determination

In relative concentration determination, Experion software provides an estimate of protein concentration by comparing the ratio of the corrected area of each protein to that of the internal standard, which is present at a single, known concentration in the sample. Experion software performs relative concentration determination for all identified protein peaks in a sample and presents the results in the result table.

By default, the Experion system performs relative concentration determination against the UM. When a user-defined internal standard is used, Experion software replaces the corrected area of the UM with that of the user-defined standard in the calculation. To facilitate comparison, it presents the recalculated concentrations in the result table. Experion software also allows manual identification of the user-defined standard if the program is not able to automatically detect it.

This single-point calibration method assumes that each protein is chemically similar to the internal standard and, therefore, that the intensity of each protein in a sample is linearly related to the intensity of the internal standard within the linear quantitation range of the Pro260 assay (2.5 µg/ml–2 mg/ml). When performing single-point calibrated methods, the internal standard serves as a reference for calculating the concentration and as a way to normalize the variations. For this reason, use of a carefully selected standard often provides better accuracy and reproducibility. This quantitation method, though it may require some work to develop a suitable standard, is a useful, convenient approach for high-throughput screening processes, since it can be easily and rapidly performed with the Experion system.

Absolute Concentration Determination

In absolute concentration determination, Experion software determines protein concentration from a multipoint calibration curve generated by a range of known concentrations of that protein. This method provides the most accurate protein concentration, but because of the requisite multipoint calibration curve, the number of samples that can be applied to a single chip is reduced, affecting sample throughput. Similar to relative concentration determination, absolute concentration may require work to develop an appropriate calibration curve.

Absolute quantitation on the Experion system provides absolute concentrations for one protein of interest; the concentrations of other proteins in the sample are calculated relative to the internal standard. Because the calibration curve statistically reduces the effects of sample variation and is usually created using the same protein being analyzed, the results obtained are often more accurate than those generated by relative concentration determination (Zhu and Strong 2006).

To generate the calibration curve, three to six known concentrations of the protein to be quantitated are loaded into different sample wells. Experion software compares the peak areas of the different concentrations to the peak area of an internal standard, plots the peak ratios as a function of concentration, performs a linear regression analysis of the calibration data, and then uses the data to calculate the protein concentrations in the samples. As with relative quantitation, either the UM or a user-defined protein can be used as the internal standard. Experion software displays the calculations for both relative quantitation and absolute quantitation, enabling easy comparison of the values achieved by these different approaches.

Before using the calibration curve to determine the absolute concentration of a target protein, it is important to ensure that the curve is linear. The r^2 is calculated by Experion software and is provided in the calibration curve dialog box. The square root of this number, or the linear correction coefficient (r), is the measurement of the linear fit. An r of 0 indicates no fit, while 1 indicates a perfect fit when the slope of the line is positive. The user must determine an acceptance value of r (0.00–1.00) for an assay by performing a series of experiments using the known

protein standards. This involves varying concentrations of the known protein to be used in the calibration curve. Many other factors can affect the quality of a calibration curve. The following practices should alleviate most of them:

- Generate the calibration curve using the same protein as the target protein. If this protein is unavailable, use a commercial protein standard as the calibrant — using a commercial protein standard to generate a calibration curve usually has a much higher chance of generating more accurate results than the single-point calibration methods (relative concentration determination)
- Confirm the linearity of the calibration curve before using it in any calculations. Determine the acceptable values of the correlation coefficient (0.00-1.00) for an assay by performing a series of experiments using the protein calibrants
- Select calibrant amounts that span the range of the concentrations expected in the sample. The range should be within the quantitative range of the assay and instrument. The lowest concentration must be below the minimum expected level in the sample, and the highest concentration must be above the maximum expected level. Often, the best results are obtained when the concentrations in the samples fall in the middle of the calibration curve
- Prepare the protein calibrant solutions by serial dilution
- Prepare a calibration curve on each chip used, to account for chip-to-chip variability
- Determine absolute concentration using the UM as the internal standard first before devoting time to developing an effective user-defined internal standard (a user-defined standard may not improve the accuracy of absolute concentration determination if no sample preparation is required prior to working with the Experion kit). If using a user-defined standard, the amount should be in the middle of the concentration range used for the calibration curve

By default, Experion software calculates absolute concentration for a single target protein. Though the software is able to use the same calibration curve to compute concentrations for different target proteins, we do not recommend this practice as each protein exhibits a unique linear response over a range of concentrations.

Conclusions

The Experion automated electrophoresis system provides options for several methods of protein quantitation along with additional advantages over conventional SDS-PAGE, including fast analysis times, reduced manual labor, and automated data analysis and storage for easy result tracking and reporting.

Protein quantitation can be performed in a variety of ways that differ in the trade-offs made between sample throughput and the degree of accuracy needed. Simple methods such as percentage determination are helpful for quick, routine sample comparisons of samples with similar protein compositions. At the other end of the spectrum, the most accurate method for protein quantitation, absolute concentration determination, utilizes not only an internal standard but a multipoint standard curve as well. Selection between these methods requires an understanding of their advantages and disadvantages and will depend on the protein samples under investigation, experimental goals, and availability of purified protein standards. The discussion provided in this article is intended to help make that decision easier.

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