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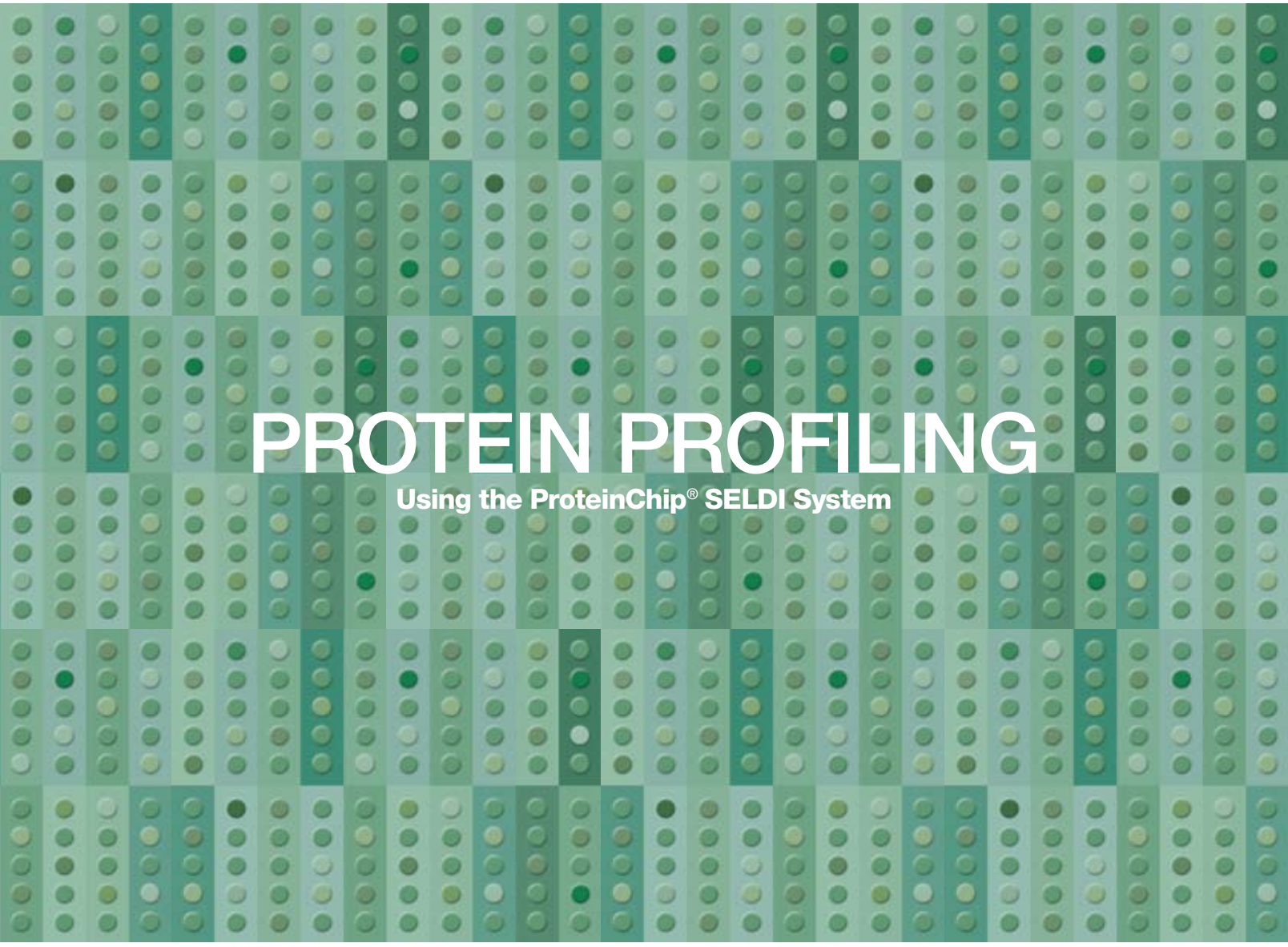

PROTEIN PROFILING

Using the ProteinChip® SELDI System

In this issue:

BIO-RAD

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BioRadiations

issue 123, 2007

TO OUR READERS

Protein biomarker discovery is a growing branch of proteomics, driven by researchers focusing their studies on those proteins likely to be involved in particular physiological conditions. A major tool for protein biomarker discovery is differential protein expression profiling, in which protein expression levels are compared across samples. Our feature article describes some of the challenges to protein biomarker discovery and how Bio-Rad's ProteinChip® SELDI system addresses those challenges.

COVER STORY

DEPARTMENTS

TECHNICAL REPORTS

Legal Notices — See page 32.

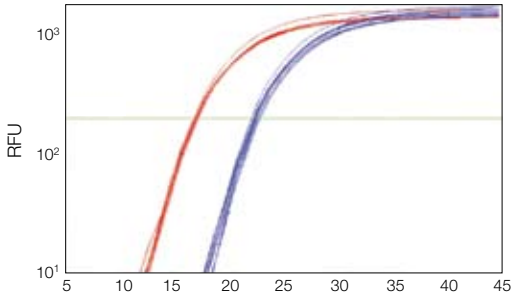
Gene Pulser® Electroporation Buffer

Gene Pulser electroporation buffer is the newest reagent to be added to Bio-Rad's line of electroporation products. The buffer is designed to emulate the natural cytoplasmic composition of cells, to minimize cell mortality while ensuring a highly efficient delivery of nucleic acids.

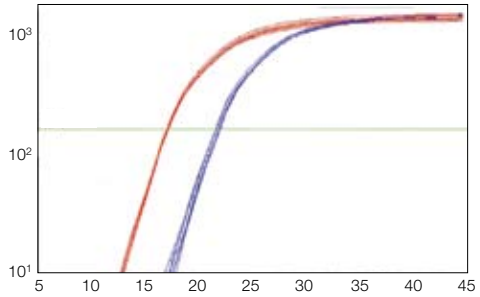
Advantages of Gene Pulser Electroporation Buffer

- Works with Gene Pulser MXcell™, Gene Pulser Xcell™, Gene Pulser II, and other electroporation systems
- Delivers both siRNA and DNA into primary cells and difficult-to-transfect cell lines
- Improves efficiency in transfection and increases cell viability after electroporation
- Can be used for transfection in a single cuvette or in a multiwell plate

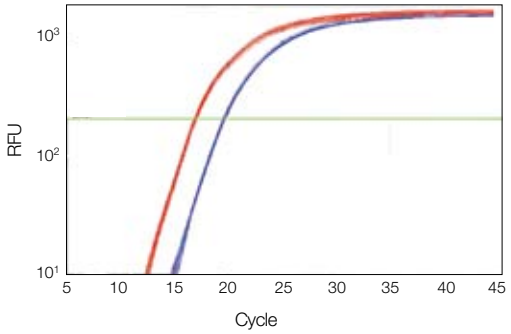
HPF cells, 96.12% silencing, 100 nM siRNA concentration



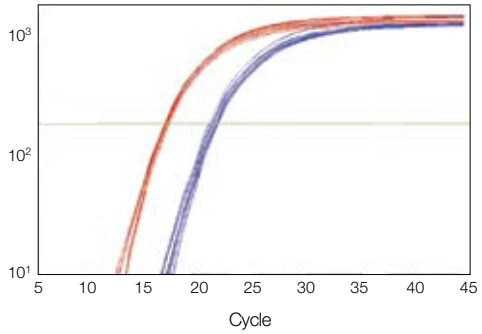
HeLa cells, 97.64% silencing, 100 nM siRNA concentration



HPF cells, 85.96% silencing, 10 nM siRNA concentration



HeLa cells, 95.26% silencing, 10 nM siRNA concentration



Demonstration of the buffer's capabilities in siRNA delivery by measuring the reduction of an endogenous gene target, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), in different cell lines. Human primary fibroblasts (HPF cells) and HeLa cells were electroporated with 10 nM and 100 nM of a siLentMer™ negative control siRNA (red) or a siLentMer GAPDH siRNA (blue). Cells were harvested 24 hr posttranscription, and RNA was isolated using the Aurum™ total RNA kit. cDNA was produced using the iScript™ cDNA synthesis kit, and qPCR was performed using the iQ™5 real-time PCR detection system. The numbers represent the reduction in transcript levels. A greater than 85% reduction in transcript levels was observed in cells exposed to GAPDH siRNA compared to cells treated with only 10 nM nonspecific siRNA. RFU, relative fluorescence units.

Ordering Information

Catalog #	Description
165-2676	Gene Pulser Electroporation Buffer, 10 x 1.8 ml
165-2677	Gene Pulser Electroporation Buffer, 30 ml

Gene Pulser MXcell™ Electroporation System

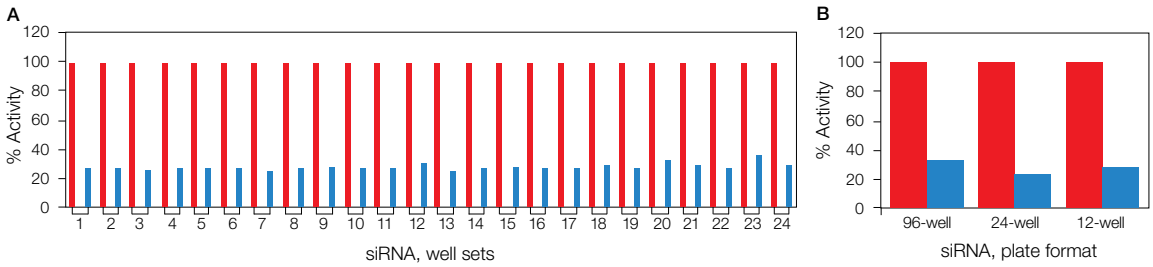
The Gene Pulser MXcell electroporation system is the newest addition to Bio-Rad's electroporation technologies. The compact system accepts several plate formats, allowing high-throughput as well as standard electroporation. The high-throughput format allows easy optimization of electroporation conditions in a single experiment, streamlining work with primary cells, difficult-to-transfect cells, and siRNA. The Gene Pulser MXcell is the only instrument capable of varying parameters, including waveform (exponential or square-wave), voltage, resistance, and capacitance, with up to 24 different protocols or well sets on a single plate. (A well set consists of four individual wells that have the same conditions applied to them.) Preset protocols with onboard help assist in setting up initial optimization conditions for any mammalian cell line.

Key Features

- Allows rapid, thorough optimization of parameters to improve transfection efficiency
- Delivers siRNA, plasmid DNA, and other biomolecules
- Works with Gene Pulser® electroporation buffer to enhance delivery of both siRNA and DNA into primary cells and difficult-to-transfect cell lines
- Accommodates multiple electroporation plate formats (96-, 24-, and 12-well) for screening or standard electroporation
- Allows programming of up to 24 different protocols on the same plate
- Establishes initial delivery conditions using preset optimization protocols
- Provides both exponential and square waveforms in one instrument
- Processes plates in 2 minutes or less
- Saves user methods for 300 programs (20 users with 15 programs each)
- Stores and recalls pulse parameters for the previous 100 protocols
- Protects against arcing
- Does not require an external power source or computer

Electroporation Plates

Electroporation plates for use with the Gene Pulser MXcell system are available in three formats: 96-well plates for high-throughput optimization conditions, and 24- or 12-well plates for standard laboratory-scale experiments. The plates produce highly uniform results with low variability from well to well. siRNA and DNA are delivered with high efficiency into mammalian cells.



Evaluation of Gene Pulser electroporation plates for uniformity and transfection efficiency. A, uniformity of a 96-well electroporation plate using luciferase siRNA transfections: percentage of luciferase activity after electroporation with 100 nM of a scramble negative control (Scr, ■) and luciferase (Luc, ■) siRNAs was evaluated in all three plate formats (96-, 24-, and 12-well). Relative light units (RLUs) from the scramble siRNA transfections were set at 100% activity, and the percentage activity of the luciferase siRNA was calculated in reference to the scramble transfections. **B**, percentage of reporter gene activity after transfection with two siRNAs, a scramble negative control (■), and a luciferase-specific siRNA (■) in CHO-Luc cells. Electroporations were performed using the three different plate formats (96-, 24-, and 12-well plates).

Ordering Information

Catalog #	Description
165-2670	Gene Pulser MXcell Electroporation System
165-2671	Power Module
165-2672	Plate Chamber
165-2681	96-Well Electroporation Plate
165-2682	24-Well Electroporation Plate
165-2683	12-Well Electroporation Plate
165-2676	Gene Pulser Electroporation Buffer, 10 x 1.8 ml
165-2677	Gene Pulser Electroporation Buffer, 30 ml

Optimization of Electroporation Conditions With the Gene Pulser MXcell™ Electroporation System

The Gene Pulser MXcell electroporation system supports the convenient plate format, allowing researchers to choose between 96-, 24-, and 12-well plates to fit experimental needs. For optimization, the 96-well plate allows testing of up to 24 different conditions. Each condition is called a well set, consisting of four individual wells that will have the same conditions applied to them. The well set allows researchers to perform high-throughput and replicate experiments. If more cells are required, 12- and 24-well plates can be used to accommodate larger sample volumes.

Factors that affect efficient electroporation of cells are waveform, pulse duration, field strength, cells and cell density, nucleic acid concentration and type, and electroporation buffer. These conditions can be tested and optimized rapidly using the Gene Pulser MXcell system.

Waveform

The two most common waveforms used in electroporation are the square and exponential (voltage decay) waveforms. The square wave relies on a charge being applied to the cells for a set time. The exponential waveform builds up a charge in a capacitor and when applied to the sample, the voltage delivered decays exponentially until the charge remaining is about 37% of the original pulse. The time over which voltage decay occurs is known as the time constant, τ.

Pulse Duration

The time constant is equal to (R x C), R being the resistance of the sample and system, and C the capacitance set on the instrument. Square waves are not associated with a time constant; instead, they are determined by the pulse duration (pulse length) — a time, in milliseconds, that is programmed into the instrument. It is possible to use short or long pulse durations when optimizing square-wave electroporations. Generally, small increments (±5 msec) lower and higher than the original pulse length are tested. Additionally, it has been observed that cells benefit from multiple, shorter pulses. For example, if the optimal pulse duration is 20 msec, further optimization may be possible by giving two pulses of 10 msec each instead.

The time constant in exponential waveforms is directly related to the resistance of the sample, the resistance programmed on the electroporator, and the capacitance setting on the electroporator. Resistance of the sample can be changed in several ways. The sample volume is inversely proportional to the resistance; therefore, decreasing the volume increases the resistance.

The ionic strength of the electroporation buffer can affect the resistance (see Table 1). The gap width (gap size or interelectrode distance) affects resistance; increasing the gap width increases the resistance. Changing the gap width also affects the field strength (see next section). Cell density may also play a role in sample resistance (see Cells and Cell Density section).

Table 1. Time constants (msec) of electroporation solutions with no cells at several volumes. These results were obtained using a protocol of 260 V, 500 µF, and 1,000 Ω on a Gene Pulser Xcell™ system with 0.4 cm gap width cuvettes. This can also be done on a Gene Pulser MXcell system using volumes within the recommended range.

Solution Volume (µl)	400	600	800
	Time Constant, τ (msec)		
Gene Pulser® electroporation buffer	72	49.5	40.7
Phosphate buffered saline (PBS)	15.3	10.8	8.2
Opti-MEM medium (Invitrogen)	13.2	9.4	7.4

Field Strength

Field strength, E, is equal to V/d, where d is the gap width. Field strength is inversely proportional to the gap width; in other words, when the same voltage is applied to cuvettes with 0.4 cm and 0.2 cm gap distances, the field strength for the 0.2 cm cuvette is double that of the 0.4 cm cuvette. For this reason, when converting conditions from one cuvette to another having a different gap distance, it is critical to consider the field strength and make the necessary adjustments to the voltage.

Cells and Cell Density

Cell density may play a role in sample resistance. Cells should be actively growing and healthy for use in electroporation experiments. It is important that the cells not be contaminated with *Mycoplasma*. Typical electroporations require cells at a density of about 1 x 10^6/ml to 5 x 10^6/ml for adherent cells and 2 x 10^6/ml to 1 x 10^7/ml for suspended cells. When sample is limiting and fewer than four wells of a well set will be used, the remaining wells must contain the same volume of the same electroporation buffer.

Nucleic Acid Concentration and Type

The transfection efficiency of electroporation can be affected by the concentration, purity, and size of the molecule used. The final concentration range for plasmid DNA is typically 5–40 µg/ml. siRNA is used at final concentrations of 10–100 nM.

Electroporation Buffer

The buffer used to electroporate mammalian cells has a direct effect on the time constant, since the sample resistance, R, is mainly due to the buffer's ionic strength. The buffer components also influence transfection efficiency and cell viability. Traditionally, a buffer with high ionic strength (low resistance), such as PBS, is used in electroporation of mammalian cells at high capacitance. Serum-free growth media has also been routinely used in electroporation with the same conditions.

Gene Pulser electroporation buffer is versatile enough to use with most cell lines, including difficult-to-transfect cells and primary cells. The buffer works well with both siRNAs and plasmid DNA, and contains components that enhance transfection efficiency and maintain overall cell health and viability. Because Gene Pulser electroporation buffer is lower in ionic strength than PBS, the time constant will be different (refer to Table 1 for an example), thus minor adjustments to the time constant are required when changing from a protocol using a different buffer.

Rapid Optimization Using Preset Protocols

The unique plate format of the Gene Pulser MXcell system allows researchers to quickly test and optimize all electroporation conditions. For example, when using Gene Pulser electroporation buffer, the capacitance can be decreased from the original protocol by the suggested 50%, while also reducing the original resistance setting by 20%. When considering electroporation of a cell line that has not been worked with, a general recommendation is to review the protocols used by several reference papers and derive a consensus starting protocol. If no references exist for a particular cell line, it is suggested that references for similar cell types be used as a starting point.

Preset protocols are available for rapid transfection optimization. They were developed based on the most commonly used cell transfection experimental parameters, and can be customized by modifying the template and saving it under a new name. For example, you can decide to optimize both exponential and square-wave pulses on the same plate, or choose to vary the exponential voltage on half of a plate, and vary the capacitance on the other as in the following example using a 96-well plate (Table 2):

Top half of plate (varying the exponential voltage):

C = 350 µF
R = 1,000 Ω
V = gradient (100–400 V)

Bottom half of plate (varying the capacitance):

V = 250 V
R = 1,000 Ω
C = gradient (200–1,000 µF)

Table 2. Plate format for sample optimization protocol.*

	1	2	3	4	5	6	7	8	9	10	11	12
A	100	100	100	200	200	200	300	300	300	400	400	400
B	100	100	100	200	200	200	300	300	300	400	400	400
C	100	100	100	200	200	200	300	300	300	400	400	400
D	100	100	100	200	200	200	300	300	300	400	400	400
E	200	200	200	350	350	350	500	500	500	1,000	1,000	1,000
F	200	200	200	350	350	350	500	500	500	1,000	1,000	1,000
G	200	200	200	350	350	350	500	500	500	1,000	1,000	1,000
H	200	200	200	350	350	350	500	500	500	1,000	1,000	1,000

* Units are V (blue) and µF (red).

Once edits have been made to the preset protocol, it is simply saved under a new file name so that it can be readily accessed. There are 21 preset protocols available on the Gene Pulser MXcell system. For information on the practical application of the optimization parameters on the Gene Pulser MXcell system, see the Technical Report in this issue (page 8).

Optimizing Surface-Enhanced Laser Desorption/Ionization Experiments

Proteomics is noted for its vast potential, but it is also noted for the difficulty in obtaining data of sufficient quality to achieve predictive value. Good proteomics mass spectral data are characterized by the number, quality, and reproducibility of observed peaks across experiments. Reproducibility is difficult to achieve not only because of the large diversity in protein expression across samples from different individuals, but also because of the large number of steps — from initial sample handling to data analysis — involved in the proteomics workflow (Figure 1). A large number of variables influence the quality of proteomics data, and optimal parameters for each step differ across sample types. The task of optimizing each of these steps can appear daunting. Furthermore, once optimized, all conditions must be kept identical across large numbers to ensure reproducibility.

The ProteinChip® surface-enhanced laser desorption/ionization (SELDI) system performs several steps of typical proteomics workflows on a single platform (Figure 1). As a result, proper optimization of the SELDI process can have a large impact on the outcome of the entire experiment. On the other hand, the use of SELDI technology can reduce the overall number of steps that require optimization. One can usually pick initial SELDI parameters that work fairly well, and roughly optimize by adjusting the two major variables of sample concentration (or dilution) and instrument laser energy. All quantitative proteomic techniques can benefit from optimization to reproducibly obtain a specific peak or spectrum of interest rather than optimizing for peak intensity in general; this article describes how to perform such optimization for the SELDI process to gain the best quality raw data. It does not discuss optimization methods such as normalization or internal sample spiking.

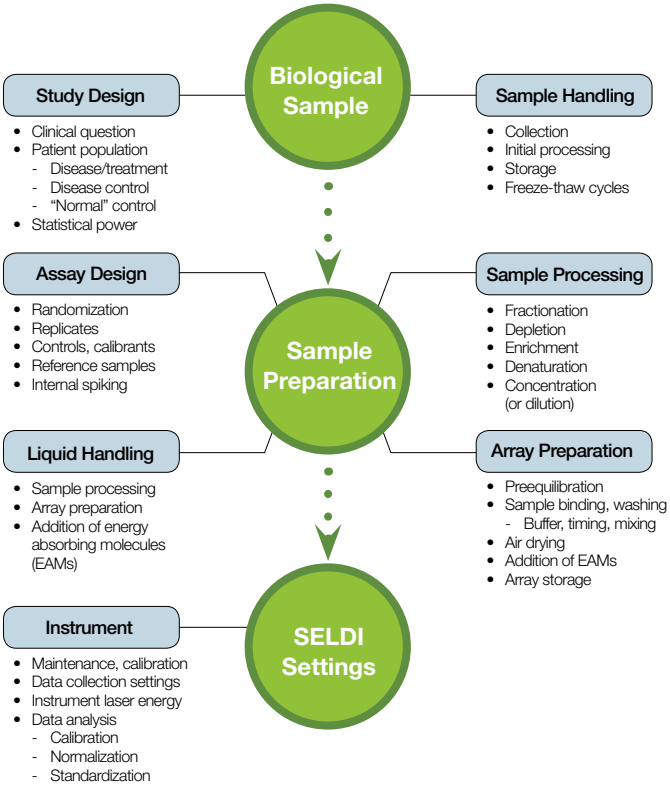


Fig. 1. Experimental variables that can affect proteomics data. Most of the steps shown are involved in all proteomics workflows, but SELDI technology performs many of them on a single platform.

Critical Spectral Defects: Peak Saturation and Peak Disappearance

Reproducibility is decreased at peak saturation (where peak intensity no longer increases with concentration) and at peak disappearance (where no peak is detected). Peak saturation and disappearance decrease the probability of discovering statistically relevant peaks by skewing the linear relationship between protein concentration and peak size. These conditions can also increase the standard deviation for peak parameters. It is important to operate in a range between peak saturation and disappearance, at a point that maximizes the number of peaks found while minimizing standard deviation in peak numbers and intensities. Both conditions can result from using suboptimal sample concentrations or laser intensity. Peak saturation occurs if either the sample concentration or the laser energy is too high, whereas peak disappearance occurs if these factors are too low. Therefore, optimizing both sample concentration and laser intensity is critical for obtaining reproducible SELDI data.

Critical Optimization Variable One: Sample Concentration

As with other proteomics tools (for example, electrophoresis gels and chromatographic columns), the total amount of protein applied to a sample should be optimized prior to beginning a SELDI experiment to avoid overloading and underloading, which can cause less reproducible results. A typical optimization involves running different dilutions of a sample under identical conditions to determine which concentration yields the greatest number of distinct unsaturated peaks (Figure 2). If there is a wide range of protein concentrations within the sample, so that some peaks disappear while others are saturated, fractionation of the sample may be required to decrease sample complexity prior to SELDI

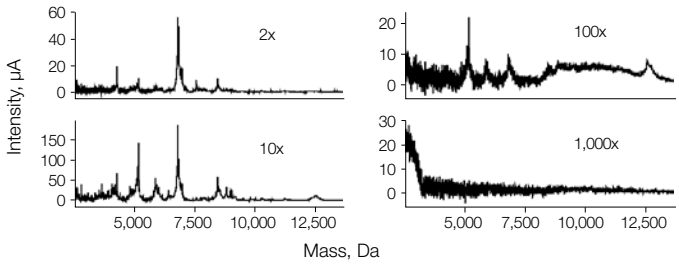


Fig. 2. Optimization of sample concentration. Different dilutions of lyophilized *Escherichia coli* lysate in binding buffer were spotted on a ProteinChip NP20 array. The numbers show the relative concentration of the sample compared to the original concentration (for example, a factor of 2x means 1/2 as much protein is present; the sample has been diluted 1:1). In this example, the 10x dilution shows the most peaks.

analysis. Fractionation will also decrease concentration. General starting points for dilution factors of biological samples are 2x, 10x, 100x, and 1,000x. The sample dilution applied affects both the number of peaks detected and spectral reproducibility (Figure 3).

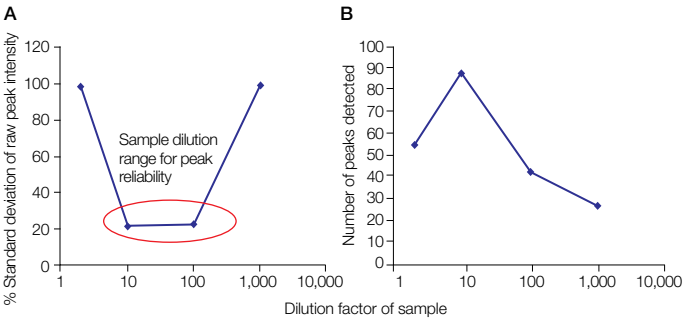


Fig. 3. Effect of varying dilution on raw peak intensity standard deviation and number of peaks detected. The data shown are plotted from the data in Figure 2. **A**, example of the effect on reproducibility of peaks within a set of raw (unnormalized) spectra. Expected linear range may be extended through the use of normalization. The smaller the percent standard deviation, the greater the reliability of the observed peaks; **B**, example of the effect on the number of peaks observed within a spectrum.

Critical Optimization Variable Two: Instrument Laser Energy

The amount of applied laser energy also affects the peak numbers and intensities and spectral reproducibility. If the amount of applied laser energy is too high, the observed peak heights no longer relate to peak concentration. If this amount is too low, some peaks will not be detected. For both conditions, peak intensities are less reproducible. To gain results with any sample that are statistically significant, applied laser energy should be optimized. Good general starting conditions for a laser power study depend on the mass range (Table 1). For the low mass range, start with 800, 1,150, and 1,500 nJ. For the high mass range, start with 3,000, 4,500, and 6,000 nJ.

Optimization Workflows

The results of an optimization study experiment (two instrument settings and five sample dilutions) are shown in Figure 4. The instrument settings, suggested initial laser ranges, and the default values for low and high masses in this experiment are listed in Table 1. It may be seen that the instrument protocol chosen and the sample dilution both affect the peak standard deviation. In this example, the standard deviation (peak variation) is minimized at 10x dilution using the high mass protocol.

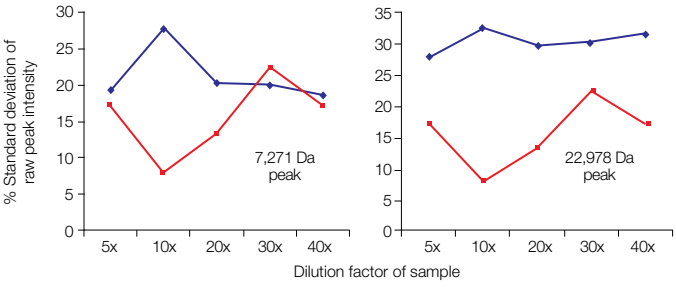


Fig. 4. Effect of varying dilution on raw peak intensity standard deviation for one sample type. Peaks were chosen arbitrarily across the mass range. Minimum standard deviation of the raw data was achieved for all chosen peaks at 10x dilution using high mass collection conditions. The blue line shows data taken using low mass collection conditions; the red line using high mass conditions (Table 1). For an actual study, this optimization would be done for each sample type and mass range of interest.

Table 1. Starting SELDI parameters for spectral acquisition.

Mass Range Parameter	Low Mass Range	High Mass Range
Mass range (Da)	0–20,000	15,000–180,000
Protein concentration (µM)	50–2,000	50–2,000
Focus mass (Da)	5,000	15,000
Matrix attenuation (Da)	1,000	5,000
Sampling rate (MHz)	800	400
High laser (nJ) (Warming shots — optional)	Low laser + 10%	Low laser + 10%
Low laser (nJ) (Data shots — optimize)	800–1,500	3,000–6,000

The best optimization strategy for SELDI experiments depends on the experiment goal. For biomarker discovery and differential expression analysis, the goal of optimization is to maximize the possibility of finding peaks of interest. Optimal conditions will maximize both the number of peaks detected and their reproducibility. Once you have selected peaks of interest and are assaying large numbers of samples, optimize conditions again to maximize reliability, resolution, and signal-to-noise ratio for the peaks of interest. In either case, parameter optimization is best done with a pooled or model sample that closely matches the samples of interest. Optimization workflows for biomarker discovery and quantitation are given below.

Optimization Workflow for Biomarker Discovery

To maximize the possibility of finding peaks of interest:

1. Run sample at various dilutions or fractionation conditions.
2. Run each sample at several different laser energies.
3. Set other collection conditions to generic values (Table 1).
4. Pick 2–5 peaks of varying intensities across the mass range of interest, and calculate the standard deviation of the intensity of these peaks.
5. Detect peaks using consistent peak detection criteria, ensuring that peak detection starts above the matrix attenuation cutoff.
6. Use the conditions that optimize peak reliability and number of peaks detected for subsequent experiments.

Optimization Workflow for Quantitation

To maximize reliability, resolution, and signal-to-noise ratio when assaying selected peaks of interest in large numbers of samples:

1. Run sample at various dilutions or fractionation conditions.
2. Run each sample at several different laser energies.
3. Set focus mass for peak of interest.
4. Analyze standard deviation of peak of interest.
5. Choose the conditions that optimize peak reliability and peak intensity for expected concentration range for subsequent assays.

Summary

It is entirely possible to obtain spectra that are reproducible and maximized in terms of spectral quality and number of peaks obtained. However, the sample handling and data collection parameters should be optimized specifically for these parameters using a pooled or model sample that closely matches the samples of interest.

— Fiona Plows, Bio-Rad Laboratories

Optimization of Electroporation Using Gene Pulser® Electroporation Buffer and the Gene Pulser MXcell™ Electroporation System

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Introduction

The transfer of exogenous nucleic acids (such as plasmids or siRNAs) into mammalian cells is an important tool for the study of gene expression and metabolic pathways. The delivery of nucleic acids into cells may be achieved with the use of chemical (for example, lipid transfection reagents and $CaCl_2$) and physical (for example, electroporation, microinjection, or particle bombardment) methods. Importantly, not all methods work effectively for all cell types.

Electroporation is a well-established method of gene transfer, with sophisticated equipment able to deliver various types of voltage pulses to cells. Electroporation is thought to temporarily destabilize cellular membranes (Heiser 2000), causing the formation of pores (Gowrishankar et al. 2000), and thereby offering an effective means of transferring nucleic acids into cells.

The Gene Pulser MXcell electroporation system is capable of delivering square-wave or exponential-decay pulses and provides preset optimization protocols. The system works with a family of plate formats (96-, 24-, and 12-well plates) to allow rapid optimization of electroporation and provide flexibility for high throughput, sample size, and replicates.

Electroporation buffers play a critical role in the transfection protocol. Gene Pulser electroporation buffer is formulated to provide high transfection efficiency, while maintaining cell integrity and viability, and can be used with any cell type, including primary and difficult-to-transfect cells, and any type of nucleic acid.

When considering electroporation of a cell line you have not worked with, look at the protocols used in several reference papers to develop a consensus starting protocol. If no references exist for a particular cell line, references for a similar cell type (that is, epithelial, fibroblast, etc.) can be used. The first step in optimizing electroporation conditions is to choose a waveform, followed by optimization of relevant parameters: voltage, pulse duration, capacitance, and resistance. In our study we demonstrate the waveform optimization process for the electroporation of human primary fibroblasts (HPF). We optimized electroporation of plasmids and small interfering (si)RNA using the Gene Pulser MXcell system and Gene Pulser electroporation buffer. The optimization criteria were simple: maximal transfection efficiency and cell viability.

Methods

Cell Culture and Transfected Materials

HPF cells (American Type Culture Collection (ATCC) #CRL-2703) were grown in medium from ATCC (Iscove's modified Dulbecco's medium with 10% fetal bovine serum). Cells were passaged 1 to 2 days prior to electroporation; they were about 75–85% confluent on the day of the experiment. The plasmid DNA used in these experiments was a luciferase expression plasmid (pCMVi-Luc).

siLentMer™ Dicer-substrate siRNA duplexes (fluorescently labeled, negative control or glyceraldehyde-3-phosphate dehydrogenase (GAPDH)-specific) were used in these experiments. After electroporation, cells were transferred into growth medium and incubated at 37°C for 24 hr. Prior to harvesting, cell viability was assessed by comparing the percentage of cells attached (and therefore viable) under different conditions.

Electroporation

Sample Handling

Plasmid DNA or the siLentMer siRNA was added to the cell suspension at the desired final concentration and gently mixed, aliquotted into the appropriate well sets of a 96-well electroporation plate, and pulsed with the Gene Pulser MXcell system. In all cases, except the cell density study, electroporation was carried out with 1 x 10^6 cells/ml in Gene Pulser electroporation buffer.

Waveform Optimization

We used the Gene Pulser MXcell electroporation system preset optimization protocol Opt mini 96 well/Exp (Table 1) to identify the best waveform. We modified this protocol to fit our experience with electroporation of HPF, and to include a previously reported exponential-decay waveform with the following electroporation conditions: 250 V, 1,000 µF, and 1,000 Ω (Ray 1995).

Table 1. Preset protocol Opt mini 96 well/Exp for waveform optimization.

	Square-Wave Conditions Well Sets* 1–3			Exponential-Decay Conditions Well Sets 4–6		
Column	1	2	3	4	5	6
Rows	A–D	A–D	A–D	A–D	A–D	A–D
Voltage	200 V	250 V	300 V	250 V	250 V	250 V
Capacitance	2,000 µF	2,000 µF	2,000 µF	350 µF	500 µF	750 µF
Pulse duration	20 ms	20 ms	20 ms	—	—	—
Resistance	—	—	—	1,000 Ω	1,000 Ω	1,000 Ω

* The Gene Pulser MXcell system is programmed by well set (a set of four rows by one column in a plate; for example rows A–D under column 1 is a well set that is displayed as ABCD1 in system programming). Each set of optimization conditions was applied to wells A–D under the corresponding column on the plate.

To illustrate how easy it is to fine-tune both square-wave and exponential-decay waveforms on the Gene Pulser MXcell system, we ran additional optimization studies. To do this, we created new protocols based on our experience with electroporation of HPF to test the following conditions:

- Square-wave voltage (200 V, 220 V, and 250 V) and pulse duration (10 ms, 15 ms, and 20 ms)
- Exponential-decay waveform voltage (200 V, 220 V, and 250 V) and capacitance (200 µF, 350 µF, 500 µF, and 1,000 µF)

- Exponential-decay waveform capacitance (350 µF and 500 µF) and resistance (350 Ω, 500 Ω, and 1,000 Ω)

The previously described optimizations are based upon changing electroporation parameters on the Gene Pulser MXcell system. However, if desired, protocols can be fine-tuned further by optimizing the electroporation volume, cell density, and nucleic acid concentration. As an example, we performed a brief optimization of cell density and plasmid concentration.

Analysis of Transfection

Cells electroporated with the pCMVi-Luc plasmid were assayed for luciferase activity. We assessed the introduction of siRNAs into HPF cells in two different ways. First, electroporation of a fluorescently labeled siRNA transfection control was used to measure transfection efficiency of siRNA by flow cytometry. Second, electroporation of the siLentMer validated 27-mer siRNA targeting GAPDH was assessed by measuring the knockdown of GAPDH using real-time PCR.

Cells electroporated with fluorescently labeled siRNA were washed with phosphate-buffered saline (PBS), trypsinized, pelleted, and suspended in PBS for analysis by flow cytometry or fluorescence microscopy. Delivery of the siLentMer siRNA was assessed by prepping total RNA from cells (Aurum™ total RNA mini prep kit), converting the mRNA into cDNA (iScript™ cDNA synthesis kit), and performing real-time PCR using specific primers and iQ™ SYBR® Green supermix on the iQ™5 real-time PCR detection system to analyze for gene silencing.

Results and Discussion

The results of preset optimization protocol Opt mini 96 well/Exp in Figure 1 show that the exponential-decay waveform is a better choice than the square-wave. We continued with the optimization process for both waveforms in parallel to illustrate how the Gene Pulser MXcell system facilitates and expedites the process.

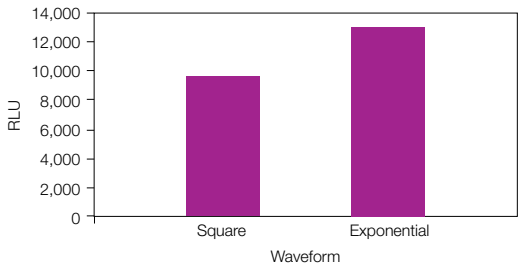
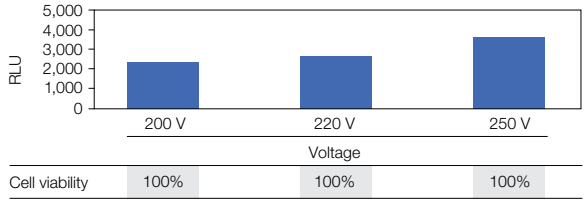


Fig. 1. Determination of electroporation waveform for HPF cells. Optimization was carried out using preset protocol Opt mini 96 well/Exp with 20 µg/ml pCMVi-Luc, assayed for luciferase activity.

The results for the optimization of square-wave electroporation shown in Figure 2 suggest that 250 V with a pulse duration of 20 ms is optimal. Although the number of pulses in this experiment did not increase transfection efficiency, we have observed that it is sometimes beneficial to divide the optimal pulse duration by two or three, and then pulse two or three times with the shorter pulse.

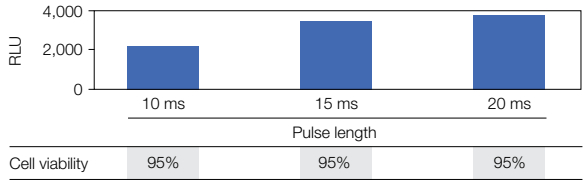
Figure 3 shows the optimization of exponential-decay electroporation results. Ignoring cell viability data, the original starting conditions appear optimal. However, cell viability was significantly better at 500 µF with no reduction in gene expression, so this would be the optimal capacitance for Gene Pulser electroporation buffer.

A. Voltage



Cell viability	100%	100%	100%

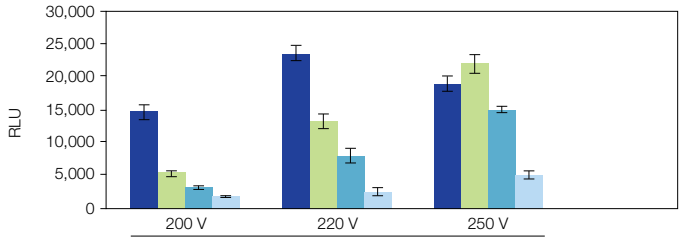
B. Pulse duration



Cell viability	95%	95%	95%

Fig. 2. Square wave optimization of voltage and pulse duration for square-wave electroporation. Luciferase activity and confluence were measured 24 hr after cells were electroporated with pCMVi-Luc using various voltages with 20 ms pulse duration (**A**), or various pulse durations at 250 V (**B**). Associated tables show resulting cell viability for each change in condition. RLU, relative light units.

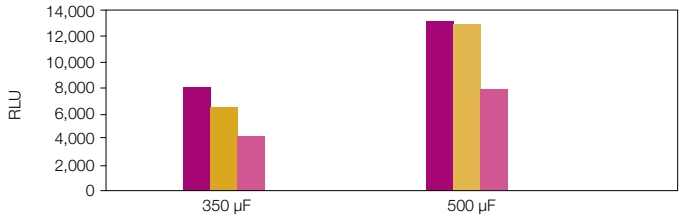
A. Capacitance



Capacitance	Voltage		
1,000 µF	85%	60%	20%
500 µF	95%	85%	70%
350 µF	98%	90%	85%
200 µF	100%	100%	95%

% Cell viability

B. Resistance



Resistance	Capacitance	
1,000 Ω	90%	85%
500 Ω	88%	75%
350 Ω	88%	75%

% Cell viability

Fig. 3. Optimization of electroporation conditions using exponential pulses. Transfection efficiency and cell viability were assessed 24 hr after HPF cells were transfected with pCMVi-Luc using varying capacitance and voltage (**A**), and then varying resistance (**B**). Associated tables show resulting cell viability for each change in condition. RLU, relative light units.

In assessing the introduction of siRNAs into HPF cells, we found that electroporation efficiency as measured by flow cytometry was nearly 100% (Figure 4). The data for electroporation of a siLentMer validated 27-mer siRNA targeting GAPDH are presented in What's New: Gene Pulser Electroporation Buffer, page 2.

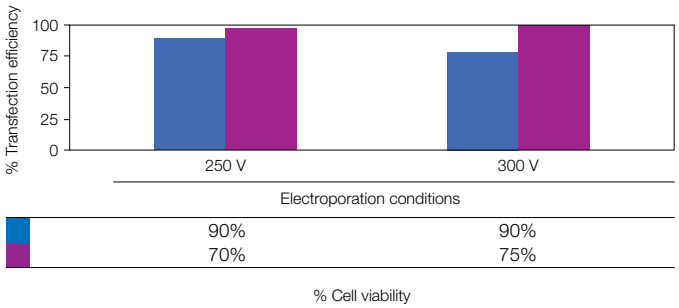


Fig. 4. HPF electroporation with fluorescently labeled siRNA using Gene Pulser electroporation buffer. Delivery of fluorescently labeled 27-mer siRNA transfection control by square-wave (■) and exponential-decay (■) pulses analyzed by flow cytometry. Associated table shows resulting cell viability for each change in condition.

From the cell density and plasmid concentration studies, we determined that using 1 x 10^6 cells/ml and 40 µg plasmid DNA/ml is optimal (Figure 5). Using 1 x 10^6 cells/ml not only resulted in much higher RLU, but also lower cytotoxicity than 0.5 x 10^6 cells/ml for both square-wave and exponential pulse electroporation. Conversely, using 2 x 10^6 cells/ml resulted in higher cell viability but lower RLU, suggesting that optimal conditions had been exceeded.

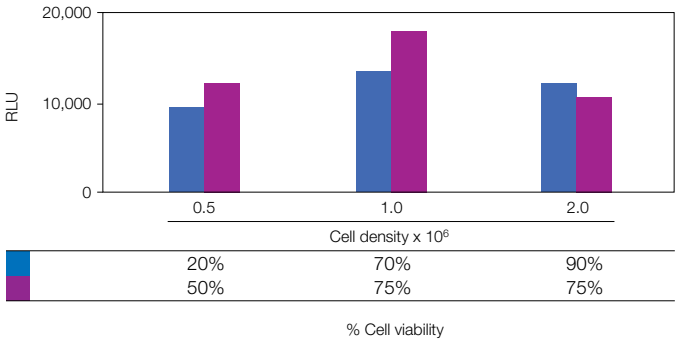


Fig. 5. Optimization of cell density for both square-wave (■) and exponential pulse (■) electroporation with 40 µg/ml plasmid DNA. Associated table shows resulting cell viability for each change in condition. RLU, relative light units.

Conclusions

Gene Pulser electroporation buffer can be used with the Gene Pulser MXcell system for effective electroporation of different nucleic acids (including plasmids and siRNAs) into many cell lines. It is possible to obtain both high efficiency and high cell viability in gene transfer experiments using Gene Pulser electroporation buffer. In addition, the multiwell plate format of the Gene Pulser MXcell system allowed multiple parameters to be tested simultaneously. For example, we were able to test both voltage and pulse duration in the same experiment. Based on the results of the optimization experiments presented here, as well as experience with other cell lines, we recommend that when beginning work with Gene Pulser electroporation buffer, test previously optimized conditions alongside conditions in which the capacitance setting is decreased to one-half or one-third of the original value. Decreasing the capacitance often increases cell viability while resulting in excellent transfection efficiency.

The general optimization workflow presented here can be followed for optimizing square-wave or exponential pulses for any cell type, and with any instrument that allows some control over electroporation conditions. Many parameters can affect electroporation; however, with the Gene Pulser MXcell system, optimization of such parameters can be accomplished easily and rapidly in a single experiment.

For more information on optimization of electroporation parameters and conditions, see Tips and Techniques, page 4.

References

Gowrishankar TR et al., Characterization of cell membrane electroporation, Center for Computational Science and Technology, http://www-fp.mcs.anl.gov/ccst/research/reports_pre1998/comp_bio/electroporation/index.html#bibitem1 (2000)

Heiser WC, Transformation of mammalian cells, pp 117–134 in Methods in Molecular Biology, Vol 130 (Tymms MJ, ed), Humana Press, Totowa, NJ (2000)*

Ray FA, Electroporation of plasmid DNA into normal human fibroblasts, pp 133–140 in Methods in Molecular Biology, Vol 48 (Nickoloff JA, ed), Humana Press, Totowa, NJ (1995)

* For a copy of this article, request bulletin RP0010.

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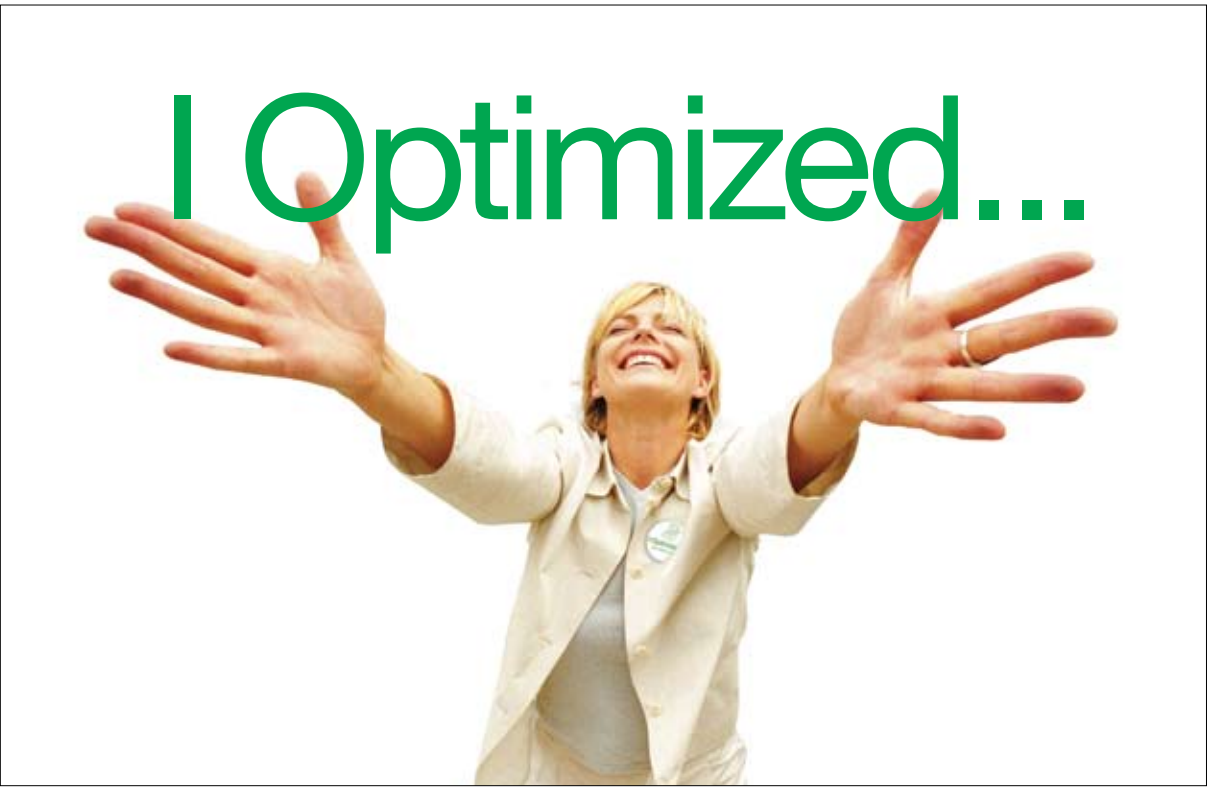

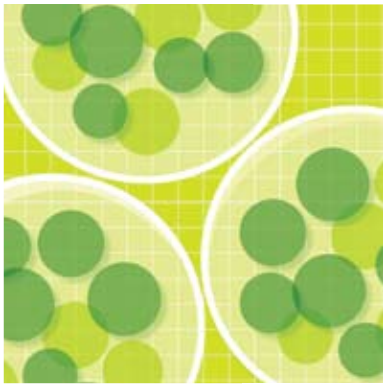

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Molecular Weight Estimation and Quantitation of Protein Samples Using Precision Plus Protein™ WesternC™ Standards and Immun-Star™ WesternC™ Chemiluminescent Detection Kit

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Introduction

Western blotting, or immunoblotting, is a widely used, sensitive technique for the detection and characterization of proteins (Burnette 1981, Towbin et al. 1979). In a typical protocol, solubilized protein is separated by SDS-PAGE and electrophoretically transferred to a nitrocellulose or PVDF membrane (Figure 1), which binds it irreversibly. This membrane blot is probed first with a primary antibody for the protein(s) of interest and then with a secondary anti-IgG antibody, which is coupled to an enzyme having an appropriate colorimetric or chemiluminescent substrate to enable detection of the primary antibody-antigen complexes on the blot.

The choice of detection substrates and documentation methods can have a great impact on the quality of the data obtained from a western blot. Chemiluminescent detection using a digital imaging system offers important advantages over film-based or colorimetric procedures, including greater sensitivity (Sandhu et al. 1991), a wider dynamic range, ease of quantitation, and the ability to vary the exposure time to optimize the signal intensity and the ratio of signal to noise on the blot.

A common objective of western blotting is to obtain quantitative information about a protein of interest, such as its estimated molecular weight (MW) or its concentration in a complex sample. Protein standards are a key component in such analyses. Standards can also serve as an internal control to monitor the progress of the electrophoresis run and the efficiency of the blot transfer step. To ensure accurate MW estimation of a protein on a western blot, standards must contain enough sharp, distinct bands of known MW that are visible following development of the chemiluminescent signal. The ability to visualize protein standards (for example, by using prestained standards) during electrophoresis and on the blot following transfer is also advantageous.

Precision Plus Protein WesternC standards are recombinant protein markers optimized for direct visualization on gels and transferred blots, as well as for parallel detection in chemiluminescent western blotting applications. The standards consist of a combination of ten prestained blue or pink bands of 10–250 kD. An integrated *Strep*-tag sequence permits detection of all bands on film or CCD-based digital imaging systems when developed with chemiluminescent substrates and a StrepTactin-HRP conjugate.

Digital CCD-based imagers, such as the Molecular Imager® ChemiDoc™ XRS system, offer the advantages of instant image manipulation, greater resolution, and a larger dynamic range than film. They perform optimally with a substrate that produces a strong

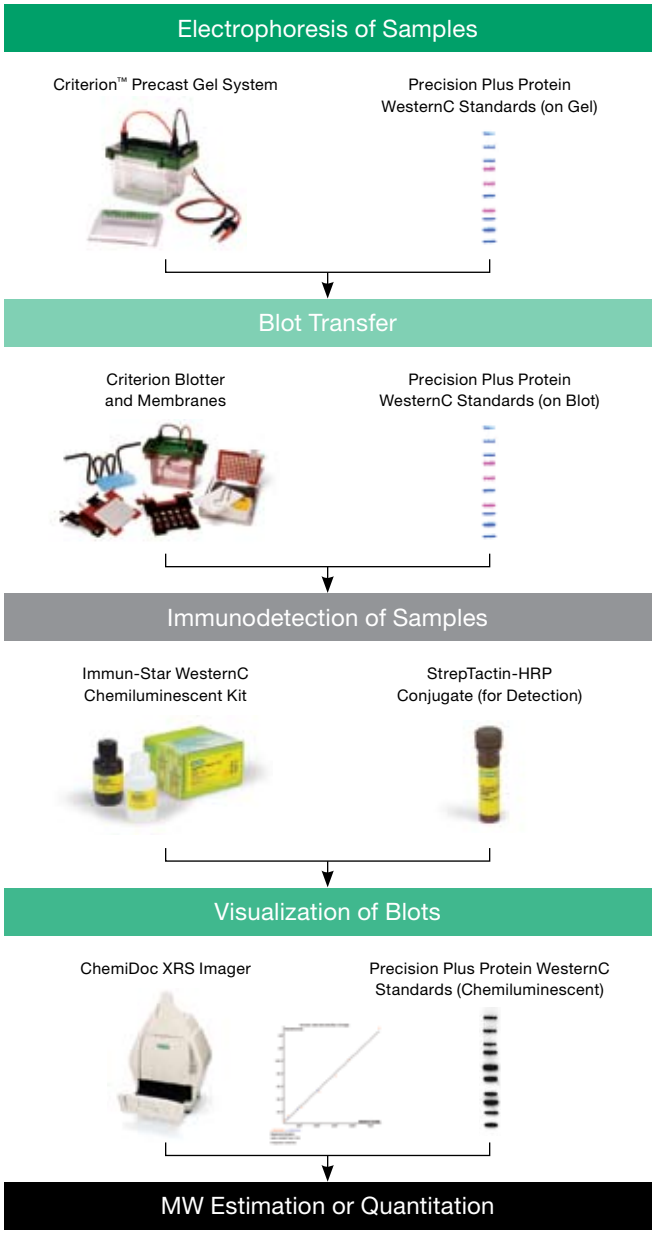

Fig. 1. Western blotting workflow from SDS-PAGE to visualization and analysis. The appearance of Precision Plus Protein WesternC standards at each step in generating a chemiluminescent western blot is shown on the right. Key equipment and reagents are shown on the left.

signal of sufficient duration. The Immun-Star WesternC detection kit provides a sensitive chemiluminescent substrate that is compatible with most horseradish peroxidase (HRP)-conjugated secondary antibodies and rapidly produces an intense signal capable of enabling detection of femtogram levels of protein for up to 24 hr, which enables the capture of digital images for qualitative and quantitative analysis.

This study demonstrates the performance and reliability of the Precision Plus Protein WesternC standards, Immun-Star WesternC chemiluminescent detection kit, and ChemiDoc XRS imaging system for estimation of the MW and concentration of protein samples in a western blotting experiment.

Methods

Sample Preparation

Purified bovine muscle actin (Sigma A3653, MW 42–43 kD) was prepared in TE buffer, pH 8.0, to a stock concentration of 1 mg/ml. A 27 kD recombinant protein was purified by immobilized metal affinity chromatography (IMAC) from a crude lysate of *E. coli* to a stock concentration of 1 mg/ml in 50 mM potassium phosphate, 300 mM sodium chloride, 20 mM imidazole buffer, pH 8.0. Dilutions of the purified actin and the 27 kD protein were prepared in Laemmli sample buffer from the stock solutions to final protein amounts of 200, 150, 100, 75, 50, 25, 12.5, 6.2, 3.1, and 1.5 ng per 5 µl gel sample load. For the quantitation experiments (unknown protein samples #1, #2, and #3), dilutions of actin and the crude *E. coli* lysate containing the 27 kD protein were prepared in Laemmli sample buffer to final target protein amounts of 50, 25, and 12.5 ng per 5 µl gel sample load. The actual amount of the 27 kD protein in the unknown samples was estimated by densitometry of Coomassie Blue-stained gels. Known amounts of the purified 27 kD protein and serial dilutions of the crude *E. coli* lysate used in this study were separated by SDS-PAGE and compared to estimate target protein in the lysate. For mass spectrometric analysis, samples were desalted into water using Micro Bio-Spin™ columns with Bio-Gel® P-6 gel. MW of the purified sample proteins was confirmed by MALDI-TOF analysis.

Gel Electrophoresis and Staining

Laemmli gel samples were heated at 95°C for 5 min, and 5 µl of sample and Precision Plus Protein WesternC standards (catalog #161-0376) were run in duplicate on Criterion 4–20% Tris-HCl precast gels in a Criterion cell at 200 V for 60 min. Following electrophoresis, one gel was blotted and the other gel was stained with Bio-Safe™ Coomassie Blue G-250 stain according to standard procedures for fixing, staining, and destaining. Stained gels were imaged on a Molecular Imager® GS-800™ calibrated densitometer using Quantity One® 1-D analysis software.

Western Blotting and Immunodetection

Following SDS-PAGE, gels were transferred to Criterion 0.45 µm nitrocellulose/filter paper sandwiches using a Criterion blotter with plate electrodes at 100 V for 30 min. Immunodetection was carried out as described in the manual included with the Immun-Star WesternC chemiluminescent detection kit. Membranes were blocked with 3% BSA (Fraction V, EMD 2930) and rinsed 3 times for 5 min. For actin immunodetection, membranes were incubated with a 1:5,000 dilution of mouse monoclonal anti-actin primary antibody (Sigma A4700), and for immunodetection of the 27 kD protein, with a 1:5,000 dilution of purified rabbit polyclonal primary antibody. Membranes were washed 5 times for 5 min and incubated with secondary antibody, a 1:50,000 dilution of goat anti-mouse (GAM)-HRP conjugate (catalog #170-5047) for actin and a goat anti-rabbit (GAR)-HRP conjugate (catalog #170-5046) for the 27 kD protein. The membranes were incubated concurrently using a 1:10,000 dilution of StrepTactin-HRP conjugate for immunodetection of the Precision Plus Protein WesternC standards. Membranes were washed 6 times for 5 min and then incubated with the Immun-Star WesternC chemiluminescent detection kit reagents at 0.1 ml/cm^2 membrane for 5 min.

Image Capture and Quantitation

Membranes were visualized on a ChemiDoc XRS system using the Chemiluminescence setting, and blot exposure times were adjusted to minimize the number of saturated pixels present in the bands used for quantitation. For MW analysis, R_f of the bands was measured using Quantity One software on the images obtained from the blots. To quantitate the amount of protein on the blot, signal volumes for actin and the 27 kD protein were measured using Quantity One software. Data were exported to Microsoft Excel software to calculate standard curves for both MW estimation (Bio-Rad bulletin 3133, 2004) and protein quantitation.

Results and Discussion

MW Analysis

A well-characterized protein of known MW, bovine actin, and a 27 kD protein were separated by SDS-PAGE alongside the Precision Plus Protein WesternC standards, transferred to blots, and visualized by chemiluminescent detection (Figure 2). R_f values for the standards were plotted versus log MW to generate a standard curve for the Precision Plus Protein WesternC standards. A linear fit to the data points was calculated, and the corresponding R^2 values for the standards on each blot are shown in Figure 3. The accuracy of the calculated MW for the sample proteins is dependent on the linearity of the standard curve generated for the markers, 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 trend line. The standard curves in Figure 3 were used to calculate the MW of actin and the 27 kD protein; the results are shown in Table 1. The differences between the calculated and mass spectrometry-determined MW were within 3% for both proteins. The accuracy of the calculated MWs confirms that the Precision Plus Protein WesternC standards provide a robust and direct method for protein MW estimation from western blots.

Table 1. Comparison of actual versus calculated MW for actin and the 27 kD protein from western blots using Precision Plus Protein WesternC standards.

Protein Sample	Actual MW (Mass Spectrometry)	Calculated MW (WesternC Standards)	Difference, %
Actin	42.49	43.77	3.0
27 kD protein	27.78	27.15	2.3

Protein Quantitation

A dilution series and three unknown dilutions of bovine actin and a 27 kD protein were separated by SDS-PAGE, transferred to blots, and visualized by chemiluminescent detection (Figure 4). Signal volumes for actin and the 27 kD protein bands were measured, and local background correction was applied to each band by normalizing to an adjacent region on the blot. The background-adjusted signal volume of each band in the dilution series was plotted against amount of protein (up to 150 ng) to generate a standard curve. A linear fit to the data points was calculated (Figure 5). The corresponding R^2 values ($R^2 > 0.99$) from the standard curves generated for both samples indicate a strong linear relationship between signal volume and amount of protein over a wide dynamic range.

Based on the standard curve from the actin dilution series, the amounts of purified actin in three dilutions were calculated using the background-adjusted volumes for each band (Table 2). Three unknown amounts of a 27 kD protein in an *E. coli* lysate sample

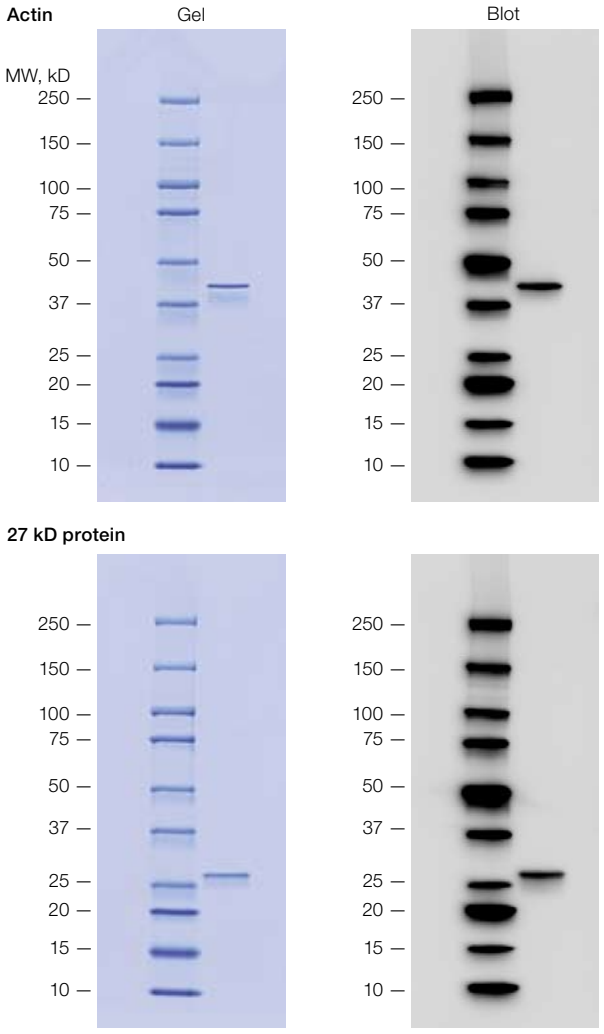

Fig. 2. SDS-PAGE and western blot analysis with Precision Plus Protein WesternC standards. Lane 1, WesternC standards; lane 2, sample: 400 ng of purified bovine actin or purified 27 kD recombinant protein. Left panels, Coomassie Blue-stained gels; right panels, chemiluminescent blots.

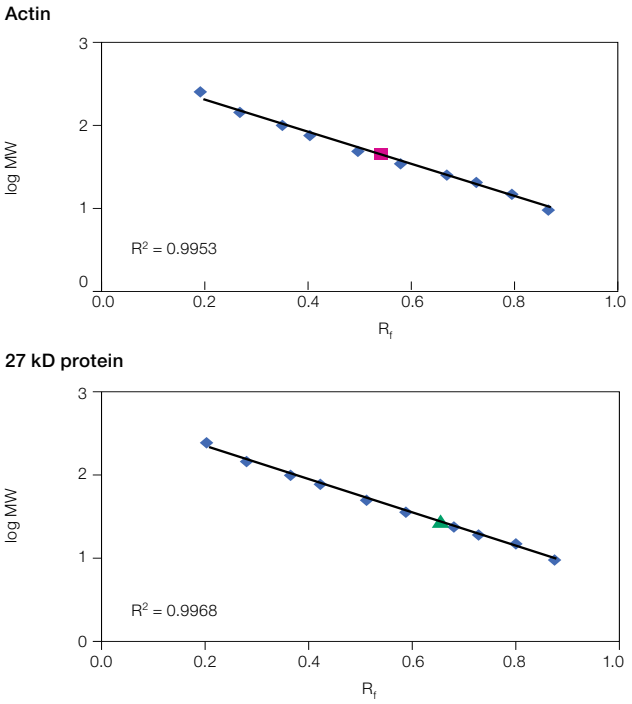

Fig. 3. Determining the MW of a protein using Precision Plus Protein WesternC standards. Standard curves of log MW versus R_f were generated using Precision Plus Protein WesternC standards from chemiluminescent-detected western blots. Linear fits to the standards' data points (10–250 kD, ◆) are shown. Curve for actin (■) blot, y = –1.97x + 2.70, curve for the 27 kD protein (▲) blot, y = –2.02x + 2.76.

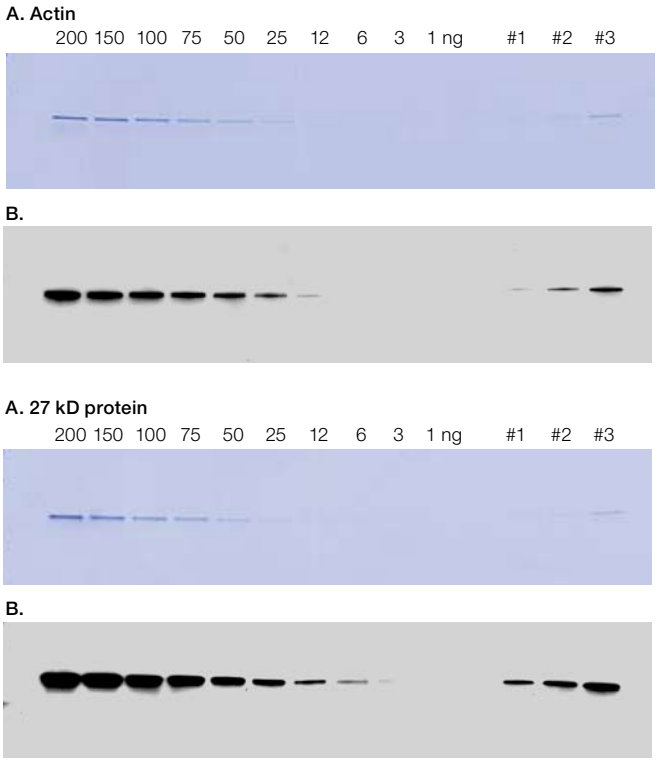

Fig. 4. Dilution series and three unknown amounts of actin and the 27 kD protein detected on chemiluminescent western blots. Samples were purified bovine actin or purified 27 kD protein and 27 kD protein from *E. coli* lysate. The amounts of protein loaded for the unknown samples were: #1, 12.5 ng; #2, 25 ng; #3, 50 ng. Panel **A**, Coomassie Blue-stained gels; panel **B**, chemiluminescent blots.

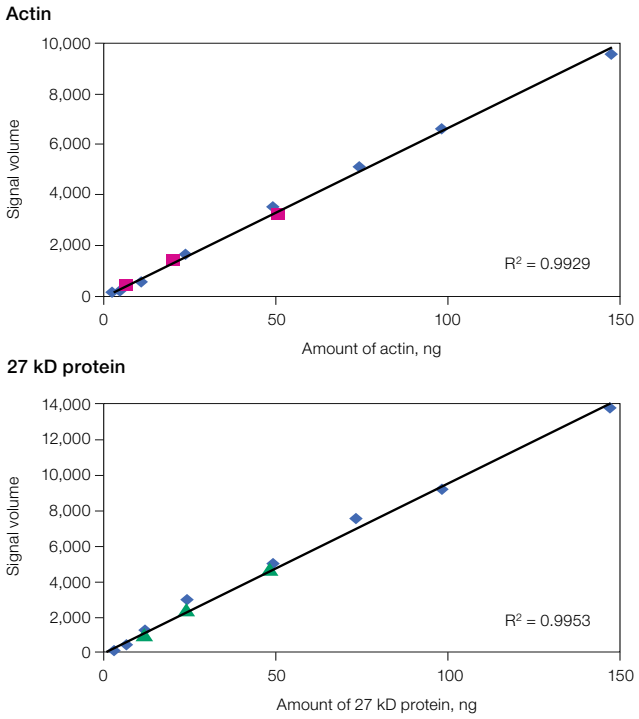

Fig. 5. Determining the linearity of protein quantitation from chemiluminescent western blots using the Immun-Star WesternC kit and ChemiDoc XRS system. Standard curves of signal vs. protein amount were generated using data from dilution series of actin and the 27 kD protein. Linear fits to the dilution series' data points (◆) are shown with the corresponding R^2 values. Upper panel, curve for purified actin dilution series (150–3.1) and the unknown dilutions of actin (■), y = 64.84x + 13.66. Lower panel, curve for purified 27 kD protein dilution series (150–1.5) and the unknown dilutions of the protein from *E. coli* lysate (▲); y = 92.56x + 231.96.

were calculated applying the same methodology to the standard curve derived from the purified form of the protein (Table 2). The accuracy of the calculated amount of the 27 kD protein in the crude lysate was within ~2% of the estimated amount. Based on the results from both proteins, the accuracy of protein quantitation was highest when the unknown samples fell within the middle of the standard curve. These results demonstrate that chemiluminescent data obtained from the Immun-Star WesternC kit and the ChemiDoc XRS system can be used to obtain reliable quantitative sample information directly from western blots.

Table 2. Comparison of actual versus calculated protein amounts for actin and the 27 kD protein from western blots using the Immun-Star WesternC chemiluminescent detection kit.

Protein Sample	Adjusted Signal Volume	Actual Protein Amount, ng	Calculated Protein Amount, ng
Actin			
Unknown #1	480	12.5	7.2
Unknown #2	1,367	25.0	20.9
Unknown #3	3,349	50.0	51.4
27 kD Protein			
Unknown #1	1,401	12.5	12.6
Unknown #2	2,572	25.0	25.3
Unknown #3	4,794	50.0	49.3

Conclusions

The performance of the Precision Plus Protein WesternC standards and the Immun-Star WesternC chemiluminescent detection kit have been tested in two typical western blotting applications. Estimation of MW from western blots is a common technique used to characterize proteins and depends upon reliable standards to obtain accurate measurements. Precision Plus Protein WesternC standards have ten sharp, prestained bands across a broad MW range that are visible at all steps of a western blotting experiment. In this study, the standards provided an accurate method of MW estimation for two independent protein samples on chemiluminescent western blots. While convenient and accurate MW estimation from blots is the principal benefit of the Precision Plus Protein WesternC standards, direct band visualization during all steps of the process is a clear advantage over unstained protein standards.

In addition to MW analysis, it is also possible to quantitate the amount of a target protein in a western blotting experiment using a known, purified control protein to construct a standard curve. This technique can be particularly useful to estimate the amount of a protein expressed in a complex sample, such as a cell lysate. Chemiluminescent detection is a highly sensitive, proven method for visualizing proteins on western blots that can take advantage of the quantitative capabilities inherent in digital imagers. The Immun-Star WesternC chemiluminescent detection kit produces an intense, enduring signal suitable for the requirements of such imagers. As demonstrated in this study, methods using the WesternC chemiluminescent substrate and the ChemiDoc XRS imager to measure signal yielded reliable estimates of target protein quantity for both a purified sample and a crude lysate.

The Precision Plus Protein WesternC standards and Immun-Star WesternC chemiluminescent detection kit thus provide a convenient and accurate method for generating high-quality, quantitative results from western blotting experiments that can be easily captured using a digital imager such as the ChemiDoc XRS system.

References

Burnette WN, "Western blotting": Electrophoretic transfer of proteins from sodium dodecyl sulfate-polyacrylamide gels to unmodified nitrocellulose and radiographic detection with antibody and radioiodinated protein A, Anal Biochem 112, 195–203 (1981)

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

Sandhu GS et al., Chemiluminescent substrates increase sensitivity of antigen detection in western blots, Biotechniques 11, 14–16 (1991)

Towbin H et al., Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedures and some applications, Proc Natl Acad Sci USA 76, 4350–4354 (1979)

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PROTEIN PROFILING AND BIOMARKER DISCOVERY

With the ProteinChip® SELDI System

Biomarkers can take a number of forms, including physiological signs (as with the body's temperature increase when fighting infection), changing-molecular concentrations in disease states (such as prostate-specific antigen in some men with prostate cancer and other prostate conditions), and abnormal molecules (as in the DNA mutations of Huntington's disease). Biomarkers are used universally as indicators of biological health.

The number of protein biomarkers is growing as researchers attempt to focus their studies on those proteins likely to be involved in particular physiological conditions. A major technique for protein biomarker discovery is differential protein expression profiling, in which protein expression levels are compared across samples. Differential expression profiling requires a technology that is not only sensitive enough to discern small differences but also able to process a sufficient number of samples to achieve statistical significance.

The ProteinChip surface-enhanced laser desorption/ionization (SELDI) system is a powerful tool for differential expression profiling. The system uses a combination of chip-based retentate chromatography and high-sensitivity mass spectrometry to create an information-rich, high-throughput discovery platform that solves many of the problems inherent in biomarker discovery.

Authors: Dominic Caseñas and Brett Houser

Biomarker Discovery and Protein Expression Profiling

Comparing protein expression levels across samples can reveal characteristic expression patterns that are correlated with particular biological states. Such comparison, called differential expression analysis or profiling, is widely used for many applications. Protein expression data are obtained through a variety of methods, from measuring the intensity of spots on a two-dimensional (2-D) gel to measuring peak intensities on a mass spectrum. Once differentially expressed proteins are identified, their expression levels can be used to classify organisms, individuals, disease states, metabolic conditions, or phenotypic responses to environmental or chemical challenges.

A key motivation for obtaining biomarkers is to increase understanding of disease pathology and thereby accelerate development of effective drugs. Biomarkers can also be helpful in disease diagnosis and prognosis and for selecting treatment options. For example, CA-125 is an ovarian cancer biomarker, and prostate-specific antigen (PSA) is a biomarker for prostate cancer. Diseases have typically been correlated with single protein biomarkers such as these.

Unfortunately, basing diagnosis on a single protein that affects or is affected by a particular disease is limiting, due to the poor sensitivity and specificity of most single biomarkers. For example, the sensitivity of CA-125 for ovarian cancer is 35% and the specificity is 98%; the sensitivity of PSA for prostate cancer is 65% and the specificity is 35%. Because of these limitations, particular focus has recently been given to uncovering panels of biomarkers for detecting a particular disease state; for example, Ciphergen's multiple-biomarker test for early detection of ovarian cancer (Wang et al. 2004, Zhang et al. 2004). The discovery of panels of biomarkers requires the use of new biomarker discovery methodologies.

Mass Spectrometry Proteomic Methods

Biomarker discovery traditionally attempts to thoroughly characterize all proteins in a sample, from identification to function. But given the enormous number of proteins present in any sample, this approach is impractical. Instead, what is needed is a method that can quickly and efficiently narrow the focus to a small number of biomarker candidates, which can be identified and then fully characterized at the molecular level. To be effective, such a method must overcome several obstacles to biomarker discovery, namely sample complexity, small changes associated with early disease states, and the need to validate predictive value.

A number of proteomics techniques are currently being used by researchers to characterize proteins. Most approaches that use mass spectrometry can be classified into two general categories: bottom-up and top-down approaches. There exists some ambiguity regarding the definitions of these methods, but the essential difference between the two has to do with expression profiling of digested versus intact proteins.

The bottom-up approach involves the digestion of proteins using a proteolytic enzyme (such as trypsin) that cleaves at well-defined sites to create a complex peptide mixture. The digested samples are then analyzed on a single platform by liquid chromatography and tandem mass spectrometry (LC-MS/MS). Differential expression using the bottom-up approach often involves labeling the sample prior to digestion with isobaric tags.

In the top-down method, quantitation and identification are separated into different processes. First, intact proteins from a complex mixture are separated using traditional separation techniques, such as liquid chromatography or 2-D gel electrophoresis, followed by differential expression analysis by spectrum analysis or gel imaging platforms. Spots or fractions that are predicted to contain biomarkers are identified using mass spectrometry.

Protein profiling using the ProteinChip SELDI platform approaches the issue of sample characterization and biomarker discovery in a different fashion (see sidebar, page 21). Complex samples are applied to chromatographic arrays for separation based on physiochemical interactions to reduce the complexity. The array is then inserted into the ProteinChip SELDI reader for measuring the mass and relative quantity of intact, undigested proteins. The reader's sensitivity* enables measurement of relatively small changes in expression level without the need for labeling. Furthermore, the reader's high throughput permits processing of large numbers of samples in each cohort to increase the statistical significance and thus assess the predictive value of a potential biomarker before identification.

Challenges of Protein Biomarker Discovery

Finding validated biomarkers that are useful in diagnosis often involves the analysis of hundreds of patient samples and requires appropriate controls. Researchers must infer the events involved in disease progression based on a number of biological "snapshots", or time points. Sample complexity and biological diversity make the interpretation of these samples difficult. Therefore, robust protein profiling techniques are needed, both to reduce sample complexity and to reproducibly measure the concentration of constituent proteins.

* The lower limit of detection is less than 10 fmol of a 150 kD protein per spot.

The ProteinChip Array — Decreasing Sample Complexity Using Retentate Chromatography

Biological samples are composed of a complex mixture of proteins and other biological molecules that are present in an extremely wide range of concentrations. For example, it has been estimated that in serum — which is a favored sample for biomarker discovery due to its easy availability — as much as 90% of the total protein mass is composed of only 1% of the total number of unique protein species (Figure 1) (Elek and Lapis, 2006). Since high-abundance proteins can mask the presence of lower-abundance proteins during profiling, separation or removal of the abundant species is essential for detecting and quantitating the majority of protein species in a sample. Therefore, decreasing sample complexity must be one component of biomarker discovery processes.

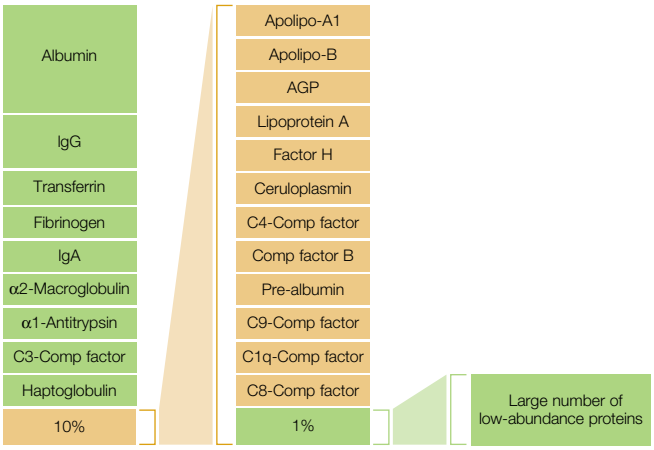


Fig. 1. A significant portion of the total protein content in serum and plasma is comprised of just a few proteins. Low-abundance proteins make up less than 1% of protein content.

In retentate chromatography, complex protein mixtures are incubated on a series of substrates, namely, ProteinChip arrays with chemically treated surfaces that provide different chromatographic properties: ion exchange, metal affinity, reverse phase, hydrophobicity, etc. (see sidebar, page 19). Different classes of proteins bind specifically to different arrays depending on their chromatographic properties, and then unbound proteins and other non-mass spectrometry compatible substances are washed away, reducing sample complexity. Thus, the arrays retain a subset of enriched proteins, which are later analyzed by mass spectrometry.

On-chip retentate chromatography has many advantages for high-throughput protein profiling. One benefit is the removal of salts and detergents that normally cause ion suppression in traditional matrix-assisted laser desorption/ionization (MALDI)-based experiments. Although typical bottom-up and top-down approaches incorporate separation techniques (such as liquid chromatography) to reduce sample complexity, ProteinChip arrays streamline chromatographic separation and differential expression analysis into a single workflow. Furthermore, because ProteinChip arrays allow separation of samples without complete digestion, analysis of intact proteins is possible.

In addition, on-chip separation requires extremely low sample volumes — ProteinChip arrays accept as little as 1 µl of sample for analysis, so many experimental conditions can be tested with each sample. Finally, by combining basic 96-well robotics, hundreds of samples can be prepared for mass spectral analysis in a few hours.

The ProteinChip Reader — High-Throughput, High-Sensitivity Mass Spectrometry

The ProteinChip SELDI system reader design is optimized to meet the needs of protein profiling and biomarker discovery. The laser desorbs and ionizes proteins bound to ProteinChip arrays, and time-of-flight (TOF) mass spectrometry is used to create a mass profile of the proteins (see sidebar, page 19). The ProteinChip SELDI reader integrates an ion source with a linear TOF mass spectrometer to create a reader that can screen intact proteins from thousands of samples with high sensitivity, without the need for digestion or tagging. The analysis of a sample run on different ProteinChip arrays and under different binding conditions results in a set of spectra that constitutes a unique protein profile from the single complex mixture (Figure 2).

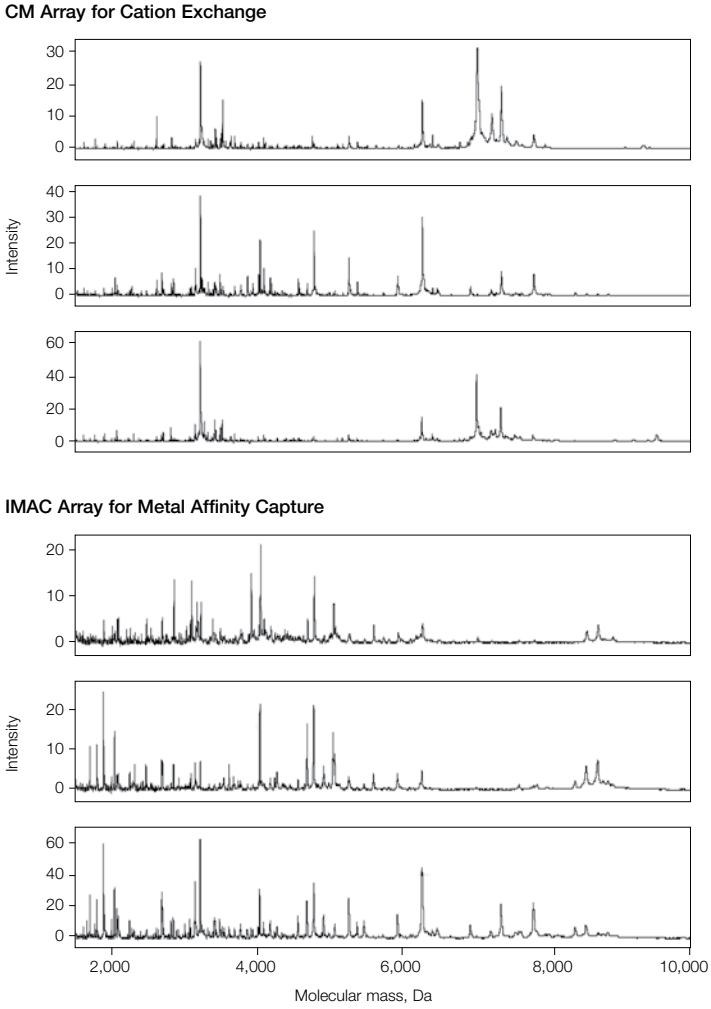
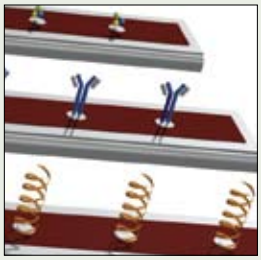


Fig. 2. The analysis of a sample run on different ProteinChip arrays and under different binding conditions results in a set of spectra that constitutes a unique protein profile from the single complex mixture. CM, carboxy-methyl; IMAC, immobilized metal affinity chromatography.

How SELDI Technology Works

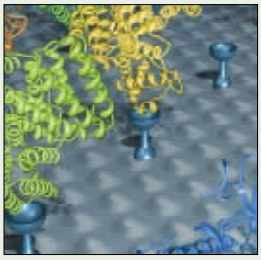
ProteinChip arrays are used to selectively bind whole classes of proteins in complex samples for detection by the ProteinChip SELDI reader. Each array consists of eight spots with chromatographic or preactivated surfaces for specific interaction with proteins of interest. Small amounts of sample are applied (usually microliter amounts of diluted sample), then selectively washed under conditions that leave behind only proteins with affinity for the surface. This process of retentate chromatography fractionates complex samples by retaining a subset of proteins while removing salts and detergents. What remains on the surface of the array is a "profile", or subset, of proteins from the original sample.

Using ProteinChip arrays involves five steps:



Step 1: ProteinChip Array Selection
ProteinChip arrays are available with different chromatographic properties, including hydrophobic, hydrophilic, anion exchange, cation exchange, and metal affinity surfaces. Arrays with higher specificity may be designed by covalently coupling protein or other bait molecules to preactivated arrays.

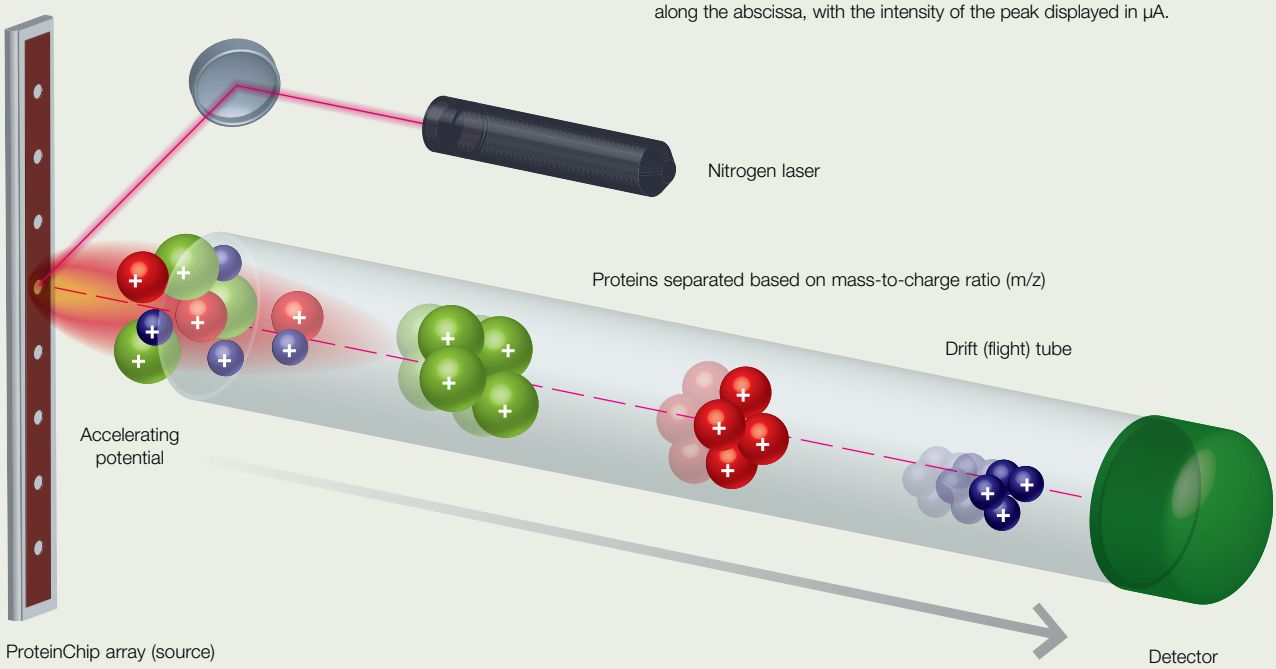
Step 2: Sample Application
Complex biological samples such as serum, cell or tissue lysates, urine, cerebrospinal fluid, or other protein homogenates, including those with high salt or detergent concentrations, can be applied directly to ProteinChip arrays. Samples are applied by manual pipetting or by liquid-handling robotics. A subset of proteins is captured by the array through simple chemical or protein-protein interactions.



Step 3: Removal of Unbound Components
After incubation, unbound proteins and other contaminants are washed from the ProteinChip array surface. Only proteins that are bound to the functional groups on the array surface are retained for analysis. These selective washes create on-chip protein (retentate) maps.

Step 4: Addition of Matrix (Energy Absorbing Molecules)
Once the sample is applied, matrix molecules are added to the array. The matrix forms crystalline complexes with the proteins and enables ionization, which is necessary for proteins to be accelerated for travel down the flight tube.

Step 5: Laser Desorption
The sample is fired upon by the laser, causing ionization and desorption of proteins from the array surface. The voltage difference at the ion source causes the now positively charged proteins to travel down the flight tube. The rate of travel is directly proportional to the mass of the charged species. Lower-mass proteins and peptides strike the detector at the other end before the higher-mass species. The intensity of a peak is proportional to both the energy of the resulting ion and the number of ions striking the detector in a given period. The spectrum produced by the detector displays the mass of the protein in Daltons along the abscissa, with the intensity of the peak displayed in µA.



Sensitivity

Since the ultimate goal of most biomarker discovery is to enable early detection and treatment of diseases, profiling ideally focuses on identifying early biomarkers. Unfortunately, identifying early biomarkers has an inherent difficulty — early stages of disease progression are typically defined by small changes in the levels of proteins of interest. Therefore, methods for biomarker discovery must be sensitive enough to detect proteins at low concentrations and to discern small differences between samples. In addition, the small differences in protein expression levels between healthy and early-disease samples necessitate running a large number of samples in order to achieve statistical significance of the differences.

One strategy to circumvent the problem of detecting lower-abundance proteins is to measure host response biomarkers instead of disease progression biomarkers. Traditional biomarker discovery experiments have focused on identifying disease progression biomarkers — those proteins that are directly involved with the disease or are produced by diseased tissue. While these biomarkers can be highly specific indicators of a particular disease, they often are expressed at such low concentrations, particularly at early disease stages, that they are not useful for early detection. In contrast, host response biomarkers, which are produced by the body as a consequence of disease, tend to be less specific to a particular disease, but they are often expressed at levels high enough to enable early detection (Fung et al. 2005). Although any single host response biomarker, such as those involved in the inflammatory response, may not be a specific indicator of a particular disease, a profile based on changes in expression levels of multiple host response biomarkers can form an effective disease indicator. Therefore, biomarker discovery methods that have the high sensitivity to allow simultaneous profiling of several proteins are advantageous.

The unique ion-source design of the ProteinChip SELDI reader improves sensitivity by minimizing the loss of ions as they enter the flight tube. Maximum transmission of ions allows the reader to obtain precise quantitation of peak intensity, which is critical for performing sample-to-sample comparisons. The increased sensitivity allows the reader to accurately measure small changes in protein expression level. In contrast to the bottom-up requirements of isobaric labeling for quantitation, the expression level of proteins using SELDI is measured with the protein still intact, allowing observation of significant changes, such as truncations or posttranslational modifications, to the amino acid sequence (Kemna et al. 2007).

The sensitivity of the ProteinChip SELDI system is also increased by an innovative detector blanking mechanism. In traditional MALDI experiments, the matrix is the largest source of ions in a given sample. These matrix ions must be added in excess, resulting in a temporary saturation of the ion detector and leading to an overall loss of detector sensitivity. The ProteinChip SELDI system allows users to create a set point in Daltons, below which interfering low molecular weight ions are effectively suppressed (Figure 3).

High Throughput

After candidate biomarkers are found, whether on the SELDI platform or any other method, many additional samples must be profiled to determine the statistical validity and predictive value of the putative biomarkers. The ProteinChip SELDI system provides the appropriate sensitivity and specificity and high-throughput validation with an accessible interface and several automated features. Introducing samples to the instrument requires simply dropping a cassette into the reader — the instrument automatically executes the appropriate protocol. The autoloader feature of the ProteinChip SELDI reader, Enterprise Edition, enables unattended acquisition of over 1,300 samples, at only 2–3 minutes per spot. In addition, the unique pass-through design of this reader allows users to add and remove additional cassettes from the reader without interrupting acquisition.

The top-down approach relies on relatively low-throughput methods for differential expression, and it has been common to pool samples to compensate, lowering statistical significance. Due to the increased complexity caused by proteolytic digestion, the throughput of the bottom-up approach is limited by the necessity to perform time-consuming separation steps prior to introduction to the mass spectrometer.

ProteinChip SELDI Software — Tools for Biomarker Discovery

In addition to sensitive high-throughput technology, the ProteinChip SELDI system includes ProteinChip data manager software, a data management and analysis software package that makes it easier to identify candidate biomarkers from data. Flexible application modules provide powerful, advanced data mining and analysis capabilities for rapid, automated analysis of multiple experiments over multiple conditions for differential expression protein profiling (Fung et al. 2005). The analysis tools include algorithms to group peaks of similar molecular weight from across sample groups of spectra into peak clusters, and to display statistical differences in the expression level of each protein in a peak cluster (Figure 3). The software also includes a robust client-server relational database system for managing and tracking large amounts of SELDI data.

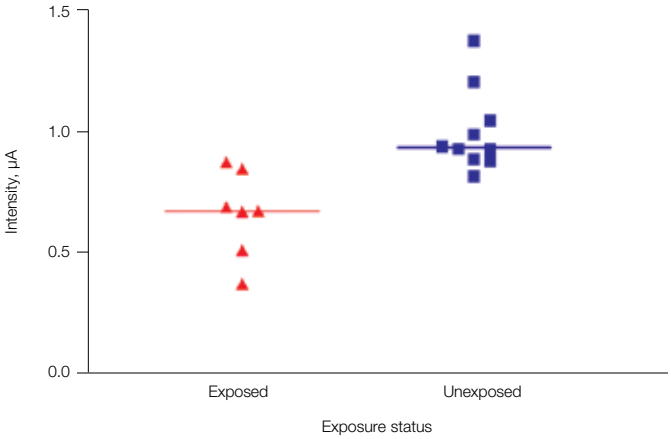


Fig. 3. Candidate marker of benzene exposure discovered using the ProteinChip SELDI system platform, later validated on an independent patient set and by SELDI immunoassay.

In addition to providing univariate statistics in the form of p-values and receiver-operator characteristic (ROC) curves, which assess diagnostic value (sensitivity and specificity), the software includes powerful multivariate tools, such as principal component analysis (PCA) and hierarchical clustering for analyzing data using a variety of methods. ProteinChip pattern analysis software, an additional software package, enables users to create a classification hierarchy of characterized proteins to create a panel of biomarkers, increasing the sensitivity and specificity over what a single marker can provide.

SELDI for Regulated Laboratories

The ProteinChip SELDI system Personal and Enterprise editions can be integrated into laboratories that require installation qualification and operational qualification (IQ/OQ). The ProteinChip OQ kit enables users to verify instrument specifications over time to ensure that the reader is operating at optimal conditions and within specifications, thereby satisfying regulatory requirements. The package includes an assortment of arrays prepared with standards and matrix for qualification of system specifications, such as resolution, mass accuracy, and sensitivity. Another package, the ProteinChip system check kit, is available for researchers looking for a low-cost option. This kit can be used for a quick check before running important experiments or between scheduled preventative maintenance visits to ensure data are collected under optimal conditions. To ensure reproducible results, each kit includes an array that automatically adjusts the gain of the ProteinChip SELDI reader. This routine helps to ensure consistent results over longer periods of time. The use of both kits ultimately improves reproducibility by ensuring data are collected under similar conditions.

Conclusion

The ProteinChip SELDI system is a sensitive, high-throughput platform for differential protein expression profiling. As such, the SELDI system addresses many of the difficulties of multiple biomarker discovery, by enabling detection of low-abundance proteins and allowing analysis of enough samples to achieve statistical significance. The system narrows the field of potential protein biomarkers to a small number of key candidates that can be further characterized using complementary technologies.

References

Elek G and Lapis K, A path or a new road in laboratory diagnostics? Biological mass spectrometry: facts and perspectives, Pathol Oncol Res 12, 178–183 (2006)

Fung ET et al., Bioinformatics approaches in clinical proteomics, Expert Review of Proteomics 2, 847–862 (2005)

Kemna EH et al., Mass spectrometry-based hepcidin measurements in serum and urine: analytical aspects and clinical implications, Clin Chem 53, 620–628 (2007)

Wang Z et al., Mass spectrometric analysis of protein markers for ovarian cancer, Clin Chem 50, 1939–1942 (2004)

Zhang Z et al., Three biomarkers identified from serum proteomic analysis for the detection of early stage ovarian cancer, Cancer Res 64, 5882–5890 (2004)

SELDI System Components

Separation — ProteinChip Arrays and Consumables

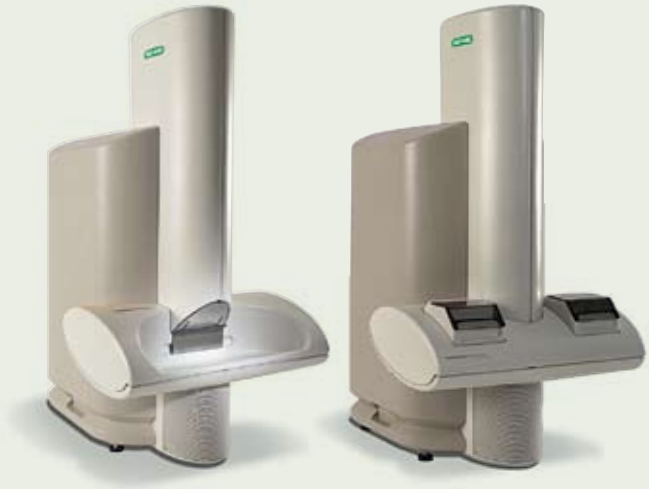
Bio-Rad offers all the consumables needed for the high-throughput analysis of samples by ProteinChip SELDI technology.

ProteinChip arrays — At the core of SELDI technology, ProteinChip arrays simplify complex samples by capturing proteins based on affinity; unbound proteins and other molecules are removed during washing.

ProteinChip buffers — Premade, quality-controlled buffers and buffer sets are designed for use with ProteinChip arrays and offer reproducible binding and washing conditions with every experiment.

ProteinChip matrix or energy absorbing molecules (EAMs) — The key to laser desorption mass spectrometry, three types of EAMs are offered, each optimized for specific applications.

ProteinChip bioprocessor — The bioprocessor enables high-throughput applications by configuring 12 individual arrays into a 96-well format that matches Society for Biomolecular Screening (SBS) standards for easy robotic integration.



Detection — ProteinChip SELDI Readers

The ProteinChip SELDI readers incorporate design features critical to the detection and precise mass calculation of proteins and peptides from prepared ProteinChip arrays. Two ProteinChip readers provide different levels of system automation and throughput:

- Personal Edition reader offers a manual feed of ProteinChip arrays, one array at a time
- Enterprise Edition reader offers an automated feed of up to 14 ProteinChip cassettes, each holding 12 ProteinChip arrays, and a built-in bar code scanner — useful features for large-scale studies requiring higher throughput

Analysis — ProteinChip SELDI Software

The ProteinChip SELDI system includes powerful software that provides a fast, effective means for organizing the large amount of data generated during biomarker studies. Custom-designed tools include up-front sample and data tracking integrated with sophisticated biostatistical analysis methods.

Comparison of Protein Phosphorylation in Cell Line and Xenograft Samples by Bio-Plex® Suspension Array and Western Blotting Techniques

Kathleen Rogers, Jeremy Fisher, Michael Lewis, Bradley Smith, and Reginaldo Prioli, Cell Signaling Technology, Inc., Danvers, MA 01923 USA

Introduction

Receptor tyrosine kinases (RTKs) mediate growth, differentiation, and developmental signals in cells by adding phosphate groups to substrate proteins to change the activation state of the proteins. When the genes encoding RTKs are altered or mutated, they can become potent oncogenes, causing the initiation and progression of a number of cancers. These pathways play a key role in the development of new drug therapies. Clinicians need to understand the degree of activation of a particular pathway and its engagement with downstream components in order to target a set of interconnected kinase-driven events along a signaling pathway. This will enable efficacious targeting of treatment.

In order to efficiently identify patients most likely to benefit from targeted drug therapy, it is essential to develop new laboratory techniques. Standard laboratory and clinical assays such as immunohistochemistry, enzyme-linked immunosorbent assays (ELISAs), and western blots can detect expression of only a limited number of proteins at once. In contrast, the Bio-Plex bead-based platform (based on Luminex technology) can detect cell-signaling events that involve up to 100 protein targets in a single sample. In a partnership, Cell Signaling Technology, Inc. (CST) and Bio-Rad Laboratories, Inc. developed, optimized, and validated Bio-Plex suspension array assays to detect and measure therapeutic targets and determinants of therapeutic efficacy.

The objective of this study was to perform Bio-Plex assays using in vitro and in vivo samples of the human non-small cell lung cancer line HCC827 (adenocarcinoma) and to compare the results to western blots.

Methods

Cell Lysate Preparation

HCC827 cells (American Type Culture Collection (ATCC)) were grown to 85% confluence and starved overnight. Cells were either untreated, treated with 100 ng/ml of epidermal growth factor (EGF, from CST), or inhibited with 1 mM gefitinib (marketed as Iressa by AstraZeneca International) for 2 hr and then treated with 100 ng/ml EGF. Cells were rinsed with phosphate-buffered saline (PBS), then lysed with Bio-Plex cell lysis buffer, sonicated three times (20 sec pulses), and centrifuged at 3,300 rpm for 10 min to remove cell debris. Supernatants were collected and the protein concentration in each sample was measured using the Bio-Rad *DC*™ protein assay.

Xenograft Lysate Preparation

We injected 1 x 10^7 HCC827 cells subcutaneously into each of ten 8-week-old female mice (Taconic Farms, Inc.). When the tumors were 1 cm^3, half the mice were administered vehicle control (100 µl Tween 80), and the remaining mice were given 150 mg/kg of gefitinib dissolved in Tween 80 by oral gavage. Tumors were harvested 24 hr after treatment. Tissue (30 mg) was excised from each tumor, placed in Bio-Plex cell lysis buffer, and homogenized by mechanical lysis. The samples were centrifuged to remove debris and the protein concentrations were measured using the Bio-Rad *DC* protein assay.

Bio-Plex Assays

Targets known to be affected by gefitinib treatment were selected for further analysis using the Bio-Plex system. The lysates (0.2 mg/ml of sample assayed in duplicate) were evaluated for total and phosphorylated ERK1/2, epidermal growth factor receptor (EGFR), and S6 ribosomal protein according to the Bio-Plex assay instructions.

Western Blotting

The lysates were evaluated for total and phosphorylated ERK1/2, EGFR, and S6 ribosomal protein using CST's total and phospho-specific antibodies. Protein (18 µg) was loaded in each well and run on a 4–20% Tris-glycine gradient gel (Jule, Inc.) using the western blot assay protocol recommended by CST (http://www.cellsignal.com/support/protocols/Western_Milk.jsp). Proteins were transferred onto nitrocellulose membrane and blocked for 1 hr with 5% bovine serum albumin (BSA) in Tris-buffered saline with 0.1% Tween 20 (TBST). Primary antibodies (CST) were diluted 1:1,000 in blocking buffer and the blots were incubated overnight at 4°C. Blots were washed three times with TBST. Goat anti-rabbit HRP-linked antibody was diluted at 1:2,000 in TBST + 5% nonfat dry milk (w/v, CST). Blots were incubated for 1 hr at room temperature, then washed three times in TBST. Blots were developed with LumiGLO and peroxidase reagents (CST). Western blots were scanned (Epson Perfection 1240 U scanner), and quantitated with Scion densitometry software (Scion Corporation).

Statistical Comparison of Bio-Plex Assays and Western Blots

We used the assay results from the nude mouse experiment to determine whether the Bio-Plex assay could detect phosphoproteins in tumor tissues. We used Student's *t*-test to statistically compare treated and control mice (considered different when $p < 0.05$). We then used Spearman's correlation test to compare the Bio-Plex assay results with western blot results obtained by Scion densitometry software.

Comparison of In Vitro and In Vivo Results With Phosphorylation Index (PI)

For comparison, we calculated a PI for each analyte in the in vitro and in vivo experiments. For each target protein, PI = (phosphoprotein MFI/total protein MFI) x 100, where MFI is the median fluorescence intensity recorded in each Bio-Plex assay. The PI normalizes the level of phosphorylation based on the total amount of protein for each analyte. The PI for each analyte was compared by determining the fold decrease in PI between the cell lysates treated with EGF or EGF + gefitinib and the control and gefitinib-treated mice.

Results and Discussion

Comparison of Bio-Plex and Western Blots Using HCC827 Cell Lysate

In a side-by-side comparison, the Bio-Plex assay and western blots showed equivalent detection of total and phosphorylated EGFR, ERK1/2, and S6 ribosomal protein (Figure 1). Both techniques showed marked reduction of phosphorylated ERK1/2, EGFR, and S6 ribosomal protein in the cell lysate from gefitinib-treated HCC827 cells compared to EGF-treated cells. Similar patterns of phosphorylation were detected by the Bio-Plex assay and the western blot, as imaged by Scion software (Figure 1).

Ability of Bio-Plex Assays to Evaluate Phosphoproteins in Solid Tumors

The Bio-Plex assays and western blots detected total and phosphorylated EGFR, ERK1/2, and S6 ribosomal protein equivalently. The Bio-Plex assays detected decreased phosphorylation of the three target proteins in mouse tumors treated with gefitinib vs. untreated controls (results using Student's *t*-test, with $n = 5$, were all $p < 0.05$; Figure 2). Densitometer-quantitated western blot results for the same comparisons are also shown in Figure 2. There was a significant correlation between the results obtained by the Bio-Plex assay and those obtained by western blotting (results using Spearman's correlation test, with $n = 30$, were $r = 0.5087$ and $p = 0.004$), confirming the two techniques perform equivalently.

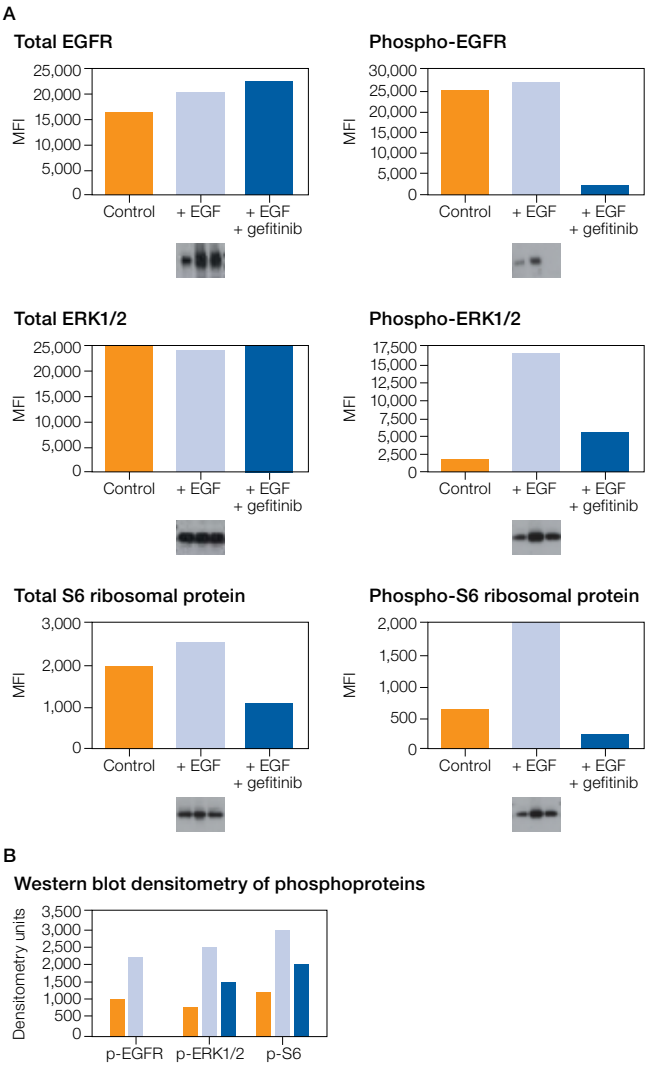


Fig. 1. Inhibited phosphorylation of three target proteins by gefitinib in HCC827 cell lysate treated with EGF. A, graphs indicate Bio-Plex assay results; images are western blots. **B**, western blots quantitated by densitometry yielded similar phosphorylation patterns to Bio-Plex assay results. The densitometry reading for p-EGFR, + EGF + gefitinib was 0. ■, control; ■, + EGF; ■, + EGF + gefitinib.

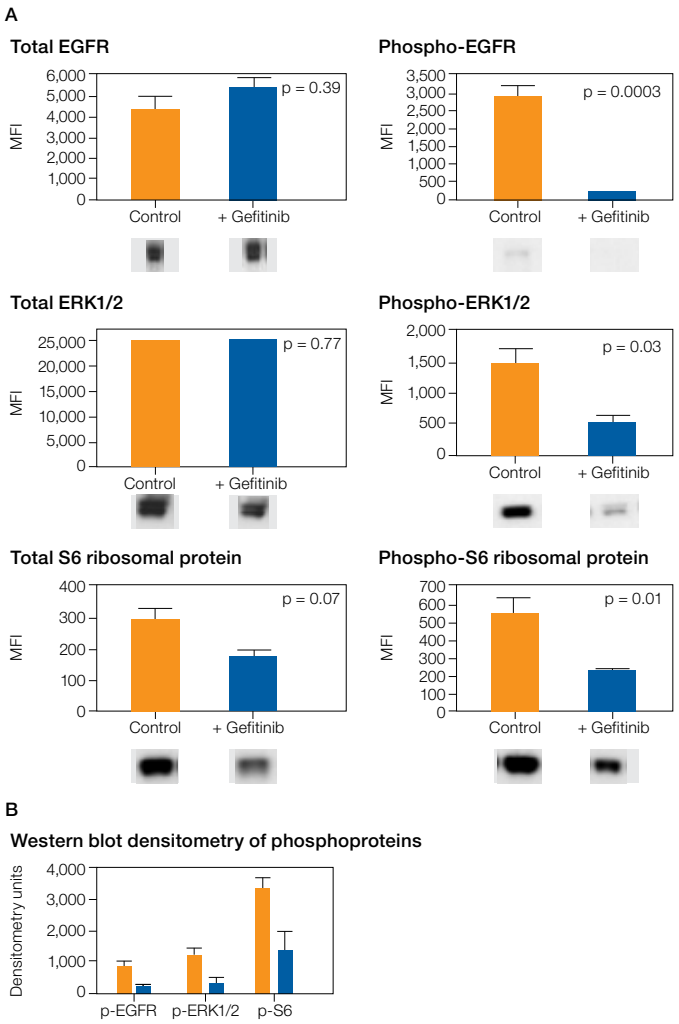


Fig. 2. Inhibited phosphorylation of three target proteins by gefitinib in HCC827 xenograft lysate from mouse tumors. A, means of median fluorescence intensity (MFI) ± the standard error of the mean (SEM) for five treated mice compared with five controls. Graphs indicate Bio-Plex assay results; images are western blots. **B**, western blots quantitated by densitometry showed the same patterns as (and correlated significantly with) Bio-Plex assay results. ■, control; ■, + gefitinib.

Phosphorylation Ratios of HCC827 Cell and Xenograft Lysates

Comparison of PI values confirmed the equivalence of in vitro and in vivo data (Table 1) and suggested that the Bio-Plex assay would be applicable for use in tissue samples.

Table 1. Comparison of HCC827 cell lysate (in vitro) and xenograft (in vivo) Bio-Plex assay data. Values represent PI, percentage of total protein MFI represented by phosphorylated protein MFI. The similar fold decrease between in vitro and in vivo phosphorylation indices indicate Bio-Plex assays would be applicable for tissue samples.

	EGFR	ERK1/2	S6
Cell lysate (in vitro)			
+ EGF	387.3	67.3	76.5
+ EGF + gefitinib	25.1	22.1	28.3
Fold decrease	15.4	3.0	2.7
Xenograft (in vivo)			
Control	63.2	6.3	179.9
+ gefitinib	4.2	2.5	119.4
Fold decrease	15.0	2.5	1.5

Conclusions

The Bio-Plex assays and western blots detected total and phosphorylated EGFR, ERK1/2, and S6 ribosomal protein equivalently in HCC827 cell lysate. Both assays' results reflected a decrease in phosphorylation upon treatment with gefitinib in vitro.

When HCC827 xenograft tumors were evaluated by Bio-Plex assays and western blots, there was equivalent detection of total and phosphorylated EGFR, ERK1/2, and S6 ribosomal protein. There was a significant correlation between the Bio-Plex assay and western blotting results. In addition, there was statistically significant suppression of phosphorylation when mice were treated with the EGF inhibitor gefitinib compared to controls.

This study also demonstrated that the in vitro and in vivo HCC827 experiments gave equivalent results and that the Bio-Plex assay is not only useful for detecting phosphoproteins in cell lysates, but also in tumor tissue.

Multiplex Phosphoprotein Assays: Detection of Downstream Epidermal Growth Factor Receptor Protein Phosphorylation and Gefitinib Inhibition in Non-Small Cell Lung Cancer Cells

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Introduction

The purpose of this study was to detect the effects of gefitinib (marketed as Iressa by AstraZeneca International) on the phosphorylation of downstream targets of epidermal growth factor receptor (EGFR; Figure 1), and to show the application of Bio-Plex® phosphoprotein assays in drug discovery based on signal transduction pathways.

The EGFR tyrosine kinase plays an important role in regulating essential cellular functions such as cell proliferation, survival, and differentiation. It has been an important targeted protein for anticancer drug discovery because inhibition of EGFR may suppress tumor growth. This anticancer effect is mediated by inhibition of phosphorylation on EGFR induced by growth factors, such as epidermal growth factor (EGF).

Gefitinib, an inhibitor of EGFR, is used to treat non-small cell lung cancer (NSCLC). The response of NSCLC to gefitinib is related to mutations occurring within the EGFR kinase domain (Sordella et al. 2004). Because Bio-Plex phosphoprotein assays (based on xMAP technology) can detect multiple phosphoprotein targets from a single cell-lysate sample, they are useful tools for revealing the phosphorylation status of the targeted protein along its signal transduction pathways.

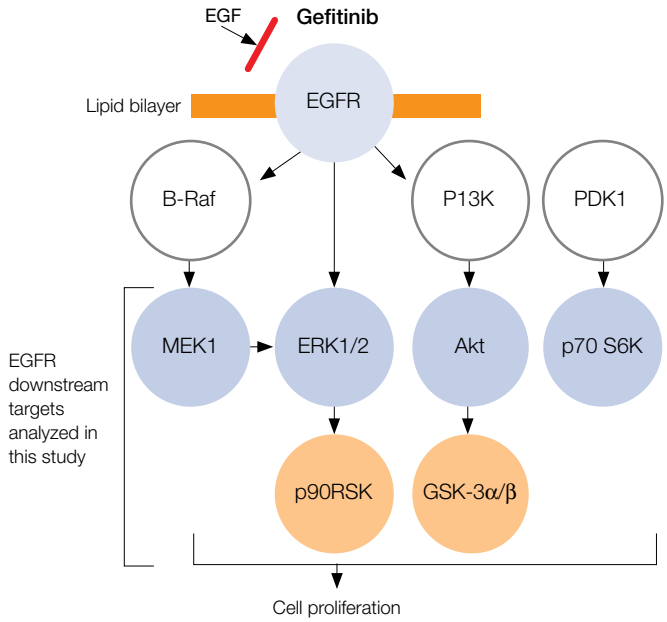

Fig. 1. Schematic of the signal transduction pathway downstream of EGFR. We used Bio-Plex phosphoprotein assays to detect the six phosphoprotein targets highlighted at bottom as well as p-EGFR. The drug gefitinib inhibits EGFR stimulation.

We used Bio-Plex phosphoprotein assays to probe the phosphorylation status of three NSCLC cell lines treated by gefitinib followed by EGF stimulation. We compared the results with western blotting results. The NSCLC cell lines we used included a wild-type strain (H-1734) and two mutated strains (H-1650 and H-1975). H-1650 contains a deletion mutation (del L747-P753) and responds to gefitinib. H-1975 has the double point mutations L858R and T790M. The cellular response to gefitinib caused by the L858R mutation is reversed by the T790M mutation. We focused on six EGFR downstream targets: p-Akt, p-MEK1, p-ERK1/2, p-GSK-3α/β, p-p70 S6K, and p-p90RSK.

Methods

Cell Culture and Cell Lysate Preparation

The three NSCLC cell lines were obtained from ATCC. All cell lines were cultured in RPMI-1640 with 10% fetal bovine serum. When cells reached about 90% confluence, we changed the culture medium to RPMI-1640 without serum and incubated the cells at 37°C overnight. Three conditions were applied to each cell line: untreated, EGF-stimulated, and gefitinib-treated/EGF-stimulated. Gefitinib was added into the serum-free medium at a final concentration of 3 µM and incubated for 3 hr, followed by EGF stimulation at 75 ng/ml for 20 min. Cells were washed and lysed with a Bio-Plex cell lysis kit according to the instructions. Total cell lysate protein concentration was measured by a Bio-Rad *DC*™ protein assay and the lysates were stored at –20°C for further analysis.

Bio-Plex Phosphoprotein Assays

The Bio-Plex p-EGFR assay was run as a singleplex assay. The assay cannot be multiplexed with other phosphoassays because it detects phosphorylated tyrosine, which is present in all other phosphoassays. To detect downstream EGFR targets, we ran a multiplex phosphoprotein assay for the six targeted phosphoproteins mentioned above. The Bio-Plex assays used 10 µg of total lysate protein for each well. Samples were tested in duplicate following the assay kit instructions. Reported results include median fluorescence intensity (MFI), standard deviation of mean MFI, and percent coefficient of variation (%CV).

Western Blot Analysis

For comparison to the Bio-Plex results, 10 µg of total lysate protein for each sample was loaded onto a Criterion™ XT Bis-Tris 4–12% gel. After transfer to a membrane, targeted protein was probed with corresponding antiphospho-epitope-specific antibodies (Cell Signaling Technology, Inc.) at room temperature overnight followed by an HRP-conjugated secondary antibody. Images were developed with chemiluminescent substrate (SuperSignal west femto substrate, Pierce Biotechnology, Inc.) and analyzed using a Molecular Imager® Gel Doc™ imaging system.

Results and Discussion

We measured tyrosine phosphorylation of EGFR and six downstream phosphoproteins in three NSCLC cell lines with and without gefitinib treatment. For all tested cell lysate samples, the MFI of Bio-Plex assays correlated very well with band intensity on western blots.

Hypothesized EGFR–Gefitinib Interactions

Gefitinib inhibits EGFR by blocking EGF from binding to it. About 10% of NSCLC patients responsive to gefitinib and almost all responsive tumors harbor EGFR mutations, but it is not clear which downstream proteins are affected (that is, are not phosphorylated) due to the effects of gefitinib on EGFR (Figure 1). Some research suggests that phosphorylation of downstream targets Akt, ERK, and STAT5 are affected (Sordella et al. 2004). We investigated some of these downstream proteins by using specific cell lines that carried EGFR mutations around several phosphorylated tyrosine motifs.

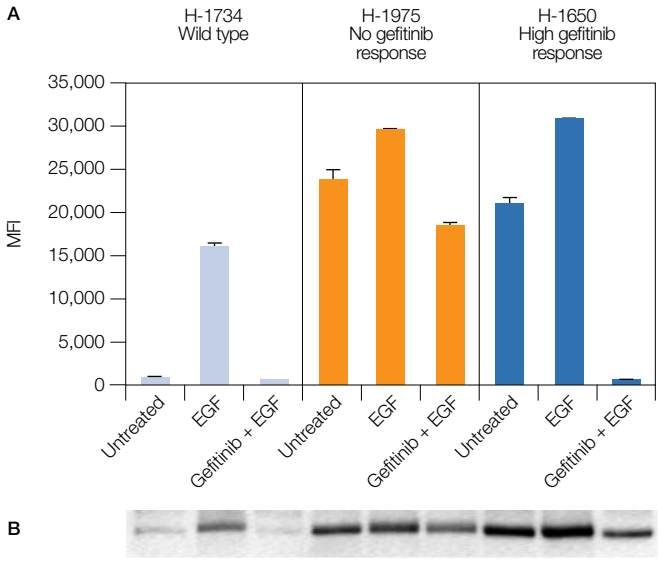

Fig. 2. P-EGFR detection on non-small cell lung cancer cell lines.
A, the Bio-Plex phosphoprotein assay detects all p-Tyr on EGFR; **B**, western blotting detects p-Tyr^{1068} on EGFR. The Bio-Plex assay compared well with western blot band intensity in detection of p-EGFR with one exception (rightmost column). Gefitinib inhibits the EGFR pathway but its effect varies among three cell cultures. MFI, median fluorescence intensity from duplicate samples. Error bars indicate standard deviations of mean MFIs.

Phospho-EGFR Detection

The Bio-Plex p-EGFR assay detected dramatic phosphorylation decreases in H-1734 and H-1650 cell lines after gefitinib treatment (Figure 2). Cell line H-1975, with a double point mutation, did not show an obvious phosphorylation decrease. Compared with the Bio-Plex data, western blotting demonstrated gefitinib inhibition on EGFR phosphorylation with H-1734 cells but much less inhibition with H-1650 cells. This discrepancy between the Bio-Plex assay and the western blot can be understood by considering the different antiphospho antibodies used in the two procedures. The Bio-Plex assay used a generalized p-tyrosine antibody, whereas the western blot used an antibody specific to the p-tyrosine-1068 epitope. EGFR contains multiple p-tyrosine sites, so the Bio-Plex assay detected overall decreases in tyrosine phosphorylation, whereas western blotting detected decreases in phosphorylation of tyrosine in one epitope only.

EGFR Downstream Phosphoprotein Detection

The results for six EGFR downstream targeted proteins are presented in Figure 3.

Six downstream targets (p-Akt, p-MEK1, p-ERK1/2, p-GSK-3α/β, p-p70 S6K, and p-p90RSK) were tested with the multiplex phosphoprotein assay using 20 µg of total cell lysate protein for duplicate data. Western blotting results required 60 µg of total cell lysate protein per sample and generated only one data point. Good correlation was demonstrated between the Bio-Plex assays and western blots in all tested samples and targeted proteins. Gefitinib inhibited the phosphorylation of all six downstream targets in NSCLC cell lines H-1734 and H-1650. In general, the inhibition was stronger on H-1650 than on the wild-type cell line, H-1734. These results suggest that the inhibition of EGFR phosphorylation by gefitinib affects multiple downstream targets involved in different signal transduction pathways. Contrary to cell line H-1650 response, the double point-mutated cell line H-1975 showed resistance to gefitinib inhibition. We saw no decreased phosphorylation on five of six downstream targets (p-Akt showed a phosphorylation decrease of about 30% after gefitinib treatment). These results are consistent with findings that the secondary mutation T790M could be responsible for drug resistance (Kwak et al. 2005).

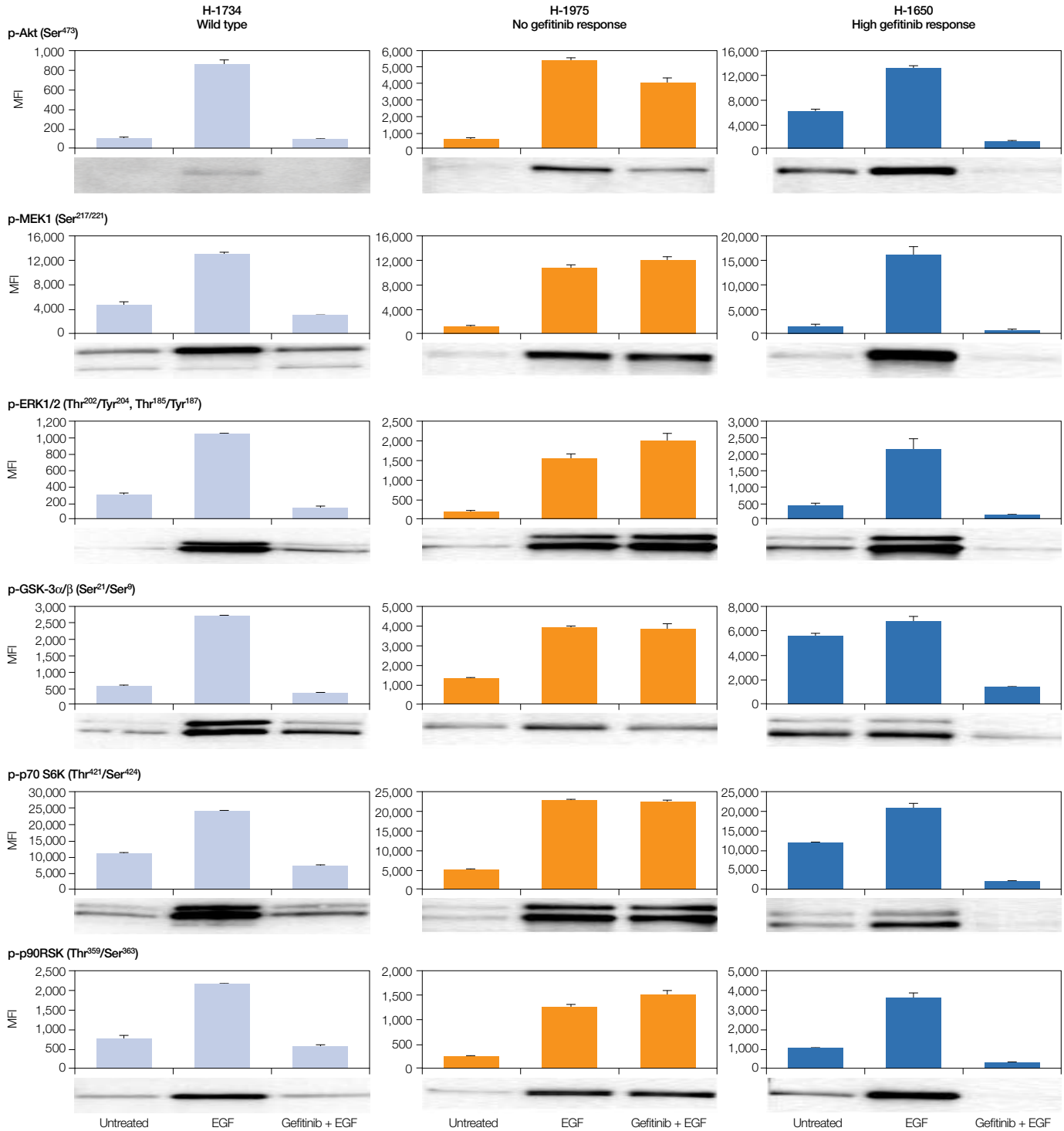

Fig. 3. Detection of EGFR downstream phosphoprotein targets. The Bio-Plex assay compared well with western blotting in detecting six phosphoprotein targets downstream of EGFR. Gefitinib inhibits the EGFR pathway but its effect varies among three cell cultures (H-1734, H-1975, H-1650). MFI, mean fluorescence intensity from duplicate samples. Error bars indicate standard deviations of mean MFIs.

Conclusion

Bio-Plex phosphoprotein assays are useful tools in studying signal transduction pathways and revealing the phosphorylation status of multiple targets in anticancer drug discovery.

References

Kwak EL et al., Irreversible inhibitors of the EGF receptor may circumvent acquired resistance to gefitinib, Proc Natl Acad Sci USA 102, 7665–7670 (2005)

Sordella R et al., Gefitinib-sensitizing EGFR mutations in lung cancer activate anti-apoptotic pathways, Science 305, 1163–1167 (2004)

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).

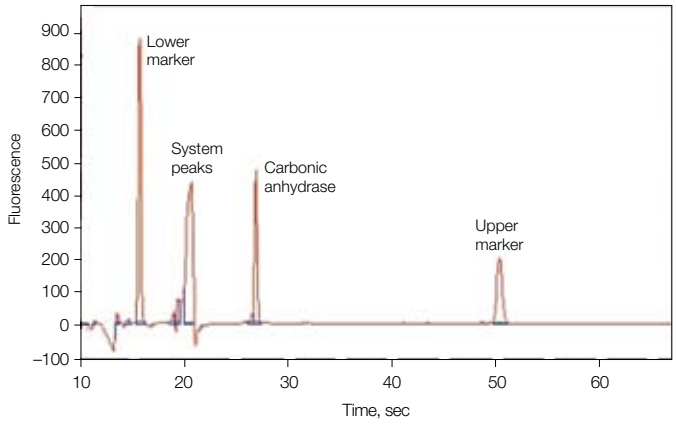

Fig. 1. Experion electropherogram. The positions of the upper marker (UM) and of a protein peak carbonic anhydrase are indicated. The UM is the default internal standard; however, carbonic anhydrase could be used as an internal standard.

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.

References

Bio-Rad Laboratories, Colorimetric protein assays, Bio-Rad bulletin 1069 (2003)

Bradford MM, A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding, Anal Biochem 72, 248–254 (1976)

Lowry OH et al., Protein measurement with the Folin phenol reagent, J Biol Chem 193, 265–275 (1951)

Zhu K and Strong W, Protein quantitation: a comparative analysis using the Experion automated electrophoresis system, Bradford and modified Lowry assays, and SDS-PAGE, Bio-Rad bulletin 5423 (2006)

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