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Multi-strip Western blotting to increase quantitative data output

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Abstract

The qualitative and quantitative measurement of protein abundance and protein modification states are essential in understanding their role in diverse cellular processes. Traditional Western blotting technique, though sensitive, is prone to produce substantial errors and is not readily adapted to high-throughput technologies. We propose a modified immunoblotting procedure, which is based on simultaneous transfer of proteins from multiple gel-strips onto the same membrane, and is compatible with any conventional gel electrophoresis system. As a result, the data output per single blotting cycle can readily be increased up to ten-fold. In contrast to the traditional “one protein detection per electrophoresis cycle”, this procedure allows simultaneous monitoring of up to nine different proteins. In addition to maintaining the ability to detect picogram quantities of protein, the modified system substantially improves data accuracy by reducing signal errors by two-fold. Multi-strip Western blotting procedure allows making statistically reliable side-by-side comparisons of different or repeated sets of data. Compared to the traditional methods, this approach provides a more economical, reproducible and effective procedure, facilitating the generation of large amounts of high-quality quantifiable data.

Keywords

protein electrophoresis; modified Western immunoblotting; high throughput; blotting errors; quantitative analysis

1 Introduction

The qualitative and quantitative measurement of protein abundance is one of the common tasks in biomedical diagnostics in the search for therapeutic targets and diagnostic or prognostic biomarkers [1] of such diseases as cancer [2-5], human immunodeficiency virus [6-8], various autoimmune disorders [9,10], Lyme [11], Creutzfeldt-Jakob diseases [12,13], syphilis [14], and many others.

Genomic methods (microarrays, PCR, SNP analysis) have many advantages, but don't directly measure the dynamics or functional state of cellular proteins. The quantitative measurement of protein modification states in response to cell stimulation, the identification of specific protein-protein interactions, establishing the contribution of unique proteins to the downstream processing of signals, and analysis of differences in protein expression levels among various cell types, is essential for understanding protein networks involved in signal transduction, linking proteins of unknown function to known cellular processes and unraveling the molecular mechanisms of specific signaling pathways and regulation of cell fate. In systems biology, the

experimental data can be used as a basis to build a comprehensive mathematical model of the system of interest [15-17]. High-quality signal and accurate quantitation of data is essential for constructing informative models of the cellular signaling system.

In studies of cell signaling the degree of protein phosphorylation in stimulated cells is compared with the basal phosphorylation level of the same protein (control). Often, the predictions of mathematical models may demand experimental data to be generated in the presence of one or more cellular perturbations, e.g. pharmacological inhibitors, exposure to physiochemical stresses or the downregulation of protein expression). Additionally variations in the dose and strength of a stimulus, measurement of detailed protein activation kinetics versus a single time-point and protein localization provide deeper insight into the threshold-dependant spatio-temporal functioning of a specific cell signaling pathway [18].

Hence, these tasks require producing large amounts of reproducible high-quality quantitative data with low variance. The processing of such high-throughput data is a costly, time-consuming multi-step procedure prone to systematic or random errors. Therefore improvements to existing experimental methods are desirable, which provide cheaper, faster and better detection of proteins [19-24].

The Western blotting technique for the immunodetection of the expression levels and phosphorylation status of electrophoretically resolved proteins [25,26] is widespread, but has several drawbacks. Here we consider the major sources of error in immunoblotting and highlight important factors that limit the efficiency of quantitative analysis. We propose a simple modification of the Western blotting procedure that increases throughput without loss of sensitivity and enables reliable side-by-side comparisons of multiple samples thus saving time, immunoblotting reagents and costly antibodies.

2 Materials and Methods

2.1 Cells, reagents and antibodies

Human epithelial lung carcinoma (A549), bladder carcinoma (T24), embryonic kidney (HEK293) and mammary epithelial (MCF-10A) cells (ATCC, Manassas, VA) were maintained in Ham's F-12, McCoy's 5A, DMEM/F-12 (all from GIBCO, Grand Island, NY) and mammary epithelium basal medium (Cambrex Biosciences, Walkersville, MD), respectively, supplemented with 10% fetal bovine serum (GIBCO, Grand Island, NY) and penicillin-streptomycin solution (100 µg/ml each) (Mediatech, Herndon, VA) in a humidified 5% CO₂ incubator at 37°C. Routine chemical reagents were obtained from Fisher Scientific (Pittsburgh, PA), unless otherwise noted. Heat inactivated BSA was purchased from Roche Diagnostics (Indianapolis, IN). Human recombinant epidermal growth factor (EGF), hepatocyte growth factor (HGF) and insulin-like growth factor 1 (IGF-1) was from PeproTech (Rocky Hill, NJ). Recombinant active MAPK was provided by Upstate (Charlottesville, VA). Wortmannin (Cell Signaling, Beverly, MA) was dissolved in dimethyl sulfoxide (DMSO) to 1 mM stock concentration and diluted to final concentration of 100 nM in cell culture medium. Bisindolylmaleimide I hydrochloride was obtained from Calbiochem (San Diego, CA). The following antibodies were used: polyclonal anti-phospho-SHP2 (Tyr542) (all from Cell Signaling, Beverly, MA), anti-GRB2 (C-23), anti-SHP2 (C-18), anti-GAB1 (H-198), anti-phospho-GAB1 (Tyr627) (all from Santa Cruz, Santa Cruz, CA), anti-phospho-EGFR (Tyr1173) (BioSource, Camarillo, CA), anti-PI3K-p85 (Upstate, Charlottesville, VA), monoclonal anti-phospho-MAPK (Thr202/Tyr204), anti-phospho-AKT (Ser473) (587F11) (both from Cell Signaling, Beverly, MA), anti-RasGAP (B4F8) and anti- α -Tubulin (DM1A) (both from Upstate, Charlottesville, VA). Prestained Precision Plus Protein standards marker was from BIO-RAD Laboratories (Hercules, CA). Blotting filter paper (2.5 mm, 7×10 cm) was purchased from E&K Scientific (Santa Clara, CA).

2.2 Cell stimulation, lysis and sample preparation

Routinely 2×10^6 cells were seeded in 60×15 mm tissue culture plates (Fisher Scientific, Pittsburgh, PA) and cultured for 48 hours before lysis. For EGF-response studies, the cells were starved for additional 16 hours in serum-free medium and stimulated with a range of EGF concentrations for different times. For inhibitor studies the cells were pretreated with 100 nM of wortmannin for 30 minutes or with 1 μ M of Bisindolylmaleimide I for 1 hour prior to stimulation. Control cells were treated with DMSO. After stimulation, the culture medium with EGF was removed, and cells were lysed in ice-cold lysis buffer (150 mM NaCl, 2.5 mM EGTA (pH 7.4), 25 mM HEPES (pH 7.4), 5% glycerol, 1% Igepal CA-630, 1% Triton X-100 (both from Sigma, St Louis, MO) containing 10 μ l/ml Phosphatase Inhibitor Cocktail Set II (Calbiochem, San Diego, CA) and one Complete protease inhibitor cocktail tablet per 10 ml (Roche Diagnostics, Indianapolis, IN). Detergent-insoluble material was removed by centrifugation at $10,000 \times g$ for 10 min at 4°C. Protein concentrations were determined using the bicinchoninic acid solution assay (Pierce Biotechnology, Rockford, IL). For SDS-PAGE, an equal amount of total protein was dissolved in 5 $\times$ Laemmli buffer (BIO-RAD Laboratories, Hercules, CA) with 5% β -mercaptoethanol and boiled for 5 min at 95°C. For LDS-PAGE (NuPAGE® in Invitrogen, Carlsbad, CA), fraction with solubilised proteins was dissolved in 4 $\times$ NuPAGE LDS Sample Buffer supplemented with NuPAGE Sample Reducing Agent (50mM DTT) (both from Invitrogen, Carlsbad, CA) and heated for 5 min at 75°C.

2.3 Electrophoresis and Immunoblotting

10 μ l of Prestained Precision Plus Protein standards was loaded in one lane. Equal volumes of prepared lysates were subjected to LDS-PAGE using Novex 4-12% gradient Bis-Tris gels for protein separation under reducing conditions in MOPS-SDS running buffer (50mM MOPS, 50mM Tris Base, 0.1% (w/v) SDS, 1mM EDTA, pH 7.7) supplemented with NuPAGE Antioxidant (all materials from Invitrogen, Carlsbad, CA) at 150V. SDS-PAGE was performed using Novex 8% Tris-Glycine gels in Tris-Glycine-SDS running buffer (25 mM Tris Base, 192 mM Glycine, 0.05% (w/v) SDS, pH 8.3) at 100V. Resolved proteins were transferred onto 0.2 μ m nitrocellulose membrane (BIO-RAD Laboratories, Hercules, CA) in XCell II Blot Module (Invitrogen, Carlsbad, CA) for 1.5 hours at 30V constant using NuPAGE transfer buffer (Invitrogen, Carlsbad, CA). The membranes were blocked with 4% BSA in Tris-buffered saline (10 mM Tris-HCl (pH 8.0), 150 mM NaCl) plus 0.5% (w/v) Triton X-100 (TBS-T buffer) for 2 hours at room temperature, then incubated with indicated primary antibodies overnight at 4°C. After extensive washing with TBS-T, the membranes were incubated with a 1:50,000 dilution of HRP-conjugated goat anti-rabbit IgG (Pierce Biotechnology, Rockford, IL) or horse anti-mouse IgG (Cell Signaling, Beverly, MA) at 1:10,000 dilution for 1 hour at room temperature and washed with TBS-T again.

2.4 Data evaluation

The immunoreactive bands were detected by KODAK Image Station 440CF after treating membranes with a working solution of the SuperSignal West Dura Extended Duration Substrate (Pierce Biotechnology, Rockford, IL) for 5 minutes. Quantitative analysis of bands was performed by KODAK Digital Science software (Kodak Scientific Imaging Systems, New Haven, CT). To enable comparison, the capture time and number of frames was equal for each separately exposed membrane or the whole membranes were exposed simultaneously. Results were plotted and statistically analyzed using Microsoft Excel program.

3 Results and Discussion

3.1 Quality versus quantity problem

The intensity of chemiluminescence emission is linearly proportional to the protein concentration over a broad range. However, the lack of linearity in the low- and high-ends of the signal makes these areas unsuitable for quantitative measurements of protein abundance. Light quenching, rapid substrate consumption and enzyme inactivation by oxidation reaction-generated free radicals leads to the saturation of a strong signal, while non-linearity of the low-end signal is due to sensitivity limitations of reaction rate accelerator (enhancer) and delayed enzyme kinetics [27,28]. Dilution of a sample can shift a strong signal to the linear range, but when the protein concentration in the sample is low, the use of more sensitive substrate (e.g. SuperSignal West Femto Maximum Sensitivity Substrate from Pierce) may increase low-end linearity and broaden the lower limit of detectability. Additionally, the loading volume per lane must be substantially increased to gain a stronger signal. If a large number of samples need to be compared, the number of lanes per gel becomes a limiting factor raising the “quality versus quantity” dilemma.

Several manufacturers (Invitrogen, Cambrex, LifeGels) offer precast mini-gels for vertical protein electrophoresis, enabling the loading of a larger number of samples per gel (e.g. 15, 17 well), resulting in apparently increased throughput. Unfortunately, thin gels with many small lanes are restricted to lower sample loading volume and consequently the sensitivity of the signal is sacrificed in order to gain higher throughput. In response to this problem the manufacturers started to offer larger and thicker midi-gels (e.g. 18, 20, 26 wells), which can accommodate a larger sample volume per lane (Invitrogen, BIO-RAD). However, the size of midi-gels dramatically augments errors caused by increased gel heterogeneity combined with unequal electrophoresis and protein transfer conditions (Fig. 1S in the Supplement).

A very helpful strategy of randomized loading was proposed to reduce correlated errors caused by gel inhomogeneity [29]. However, this approach does not eliminate the major source of errors, i.e. electrophoresis and immunoblotting procedures that involve many factors: sampling variance [30], quality and age of the gel (Fig. 2S in the Supplement), composition of buffer systems [19,20,31-33], type of blot module, materials for electroblotting (e.g. firmness of filter paper, shape of fiber pads, type of the membrane support (nitrocellulose or PVDF) [34,35]), optimized time of protein transfer onto the membrane [19,22], quality and dilution of antibodies to optimize signal-to-noise ratio, membrane washing [21], signal detection [26] and normalization of raw data [36].

3.2 Multi-strip Western blotting procedure

The presence of numerous sources of errors makes it difficult to produce a large number of high quality quantifiable data points. The simple and practical procedure, which would improve immunoblotting accuracy, maintaining high sensitivity, is necessary to solve “quantity versus quality” problem.

To this end, Swank and colleagues proposed a novel method of loading even more samples onto self-prepared mini-gels, using handmade 30-, 40-, 60- or 66-lane loaders [24]. Their system showed up to ten-fold increase in sensitivity of Western blots using sub-microgram quantities of protein when compared to conventional 15-lane gels. However, gel preparation and hand construction of special loaders for each gel is tedious, time-consuming and not as accurate as it would be in standardized commercial form. It is not clear if the demonstrated system is sensitive enough to detect less than 500 ng of loaded protein.

Here we developed a novel modification of Western blotting procedure, which, compared to the previously mentioned system, maintains high sensitivity with limits of detection of 1000

to 2500 fold smaller amounts of protein, does not require any additional tools and functions using any conventional gel electrophoresis systems. Although the following modification protocol is described for Novex 4-12% gradient NuPAGE mini-gels using prestained Precision Plus Protein marker (see Materials and Methods), it can be applied to any type of preferred mini- or midi-size gels and prestained protein size markers.

Several sets of samples are loaded onto six gels (Fig. 1A – A to F) for electrophoresis (up to twelve gels may be used). Protein molecular weight marker is loaded onto the first and/or the last lane of each gel. Separation should be started with an interval of 5-10 minutes between tandem electrophoresis units to reserve enough time for follow-up steps. When a dye line reaches the bottom of gel A, protein separation is stopped and the gel cassette is gently opened with a gel knife. The gel should remain on one of the plates. Then a centimeter-scaled transparent ruler is firmly positioned near the edge lane with a separated marker, so that zero (0 cm) aligns with the dye line and the distance from the dye line to the center of each marker band (H) is measured in millimeters (Fig. 1B). The distance between two marker bands corresponds to the migration range of certain molecular weight proteins. For example, the distance between H₂₅₀ and H₁₅₀ defines a migration range of various proteins with molecular size between 150 and 250 kDa and is shown as zone ① in Fig. 1B. The strip, which covers an area with a protein of interest located in the middle, can be precisely cut out from the gel using the gel knife. Each unique sample may provide up to the nine protein-containing areas that may be simultaneously cut out from the same gel. The number of strips to be cut out from one gel depends on the number of distinct proteins to be detected. The most frequently studied signal transduction proteins of various migration zones are listed in Table 1, which also indicates the appropriate areas to be cut out of the gel. For example, phosphorylated AKT has a molecular weight of 60 kDa and may be readily separated from activated 95 kDa insulin receptor and both 42 kDa ERK1 and 44 kDa ERK2 isoforms by cutting the gel into three strips at 22, 29, 38 and 44 mm from the dye line. The gel outside the strips is discarded, and the plate with remaining strips is covered with moistened filter paper. Similarly, identical strips from other gels (B through F) are prepared.

To avoid confusion, we assigned a code for each strip: gel C zone ⑤ indicates that the strip was derived from gel C and contains proteins with molecular sizes between 37 and 50 kDa; gel D zone ④ ⑤ – was cut out from the gel D and contains proteins with molecular sizes between 75 and 37 kDa, etc. If a protein of interest lies in the intersection of marker-defined zones (e.g. 100 kDa GAB1 protein, which migrates between zones ② and ③), the cutting area can be wider.

In the next step, the strips derived from different gels are combined onto the same assembling filter paper. The maximal number of strips that can be placed onto one assembling filter depends on its size (height and width). Routinely we use 2.5 mm thick 7×10 cm filter paper, which provides space for twelve gel strips of 0.6 cm height each. The plate with gel A strips is flipped and gently lifted so that all strips would stick to the moistened filter paper. A gel knife can be used if the strips do not independently detach from the plate. After the first strip (e.g. containing zone ① proteins) is transferred onto the assembling filter paper, the filter with remaining strips is returned onto the plate by flipping it back. Similarly, the first strip from the plate with gel B, gel C and other plates is sequentially transferred onto the assembling filter so that the strips would lay side by side and parallel to each other. When all zone ① strips are combined onto the assembling filter paper, it is placed on the fiber pads in the transfer unit, covered with a piece of nitrocellulose membrane, other moistened filters and fiber pads. Fig. 1C shows the schematic picture of the resulting blot after transfer and immunoblotting for protein of interest as described in “Materials and Methods” section. The second strip (e.g. containing zone ② ③ proteins) is similarly assembled and transferred onto the appropriate membrane (Fig. 1D). The same procedure is performed with the remaining strips.

There are, of course, other possible combinations of laying strips onto the assembling filter. Fig. 1E shows the bands of two different zone proteins transferred onto the same membrane. This situation is especially favored for the detection of phosphorylated/non-phosphorylated and housekeeping proteins (e.g. tyrosine phosphorylated 66, 52 and 46 kDa SHC isoforms, which co-migrate in zone ④ ⑤, normalized by total protein level of 28 kDa GRB2 from zone ⑥). Similarly, Fig. 1F illustrates the bands of three different zone proteins transferred onto the same membrane (e.g. serine-phosphorylated 60 kDa AKT from zone ④ and serine-phosphorylated 45 kDa MEK1/2 from zone ⑤ both normalized by total protein level of 37 kDa GAPDH from zone ⑥). In these cases, the blocked membrane can be incubated with the mixture of primary antibodies or can be cut into pieces and incubated with appropriate primary antibodies in separate dishes. Such membrane pieces may be imaged all together (e.g. phospho-AKT, phospho-MEK and GAPDH), in combination (e.g. the first piece: phospho-AKT and phospho-MEK, the second piece: GAPDH) or separately.

3.3 Increased data throughput using multi-strip Western blotting procedure

The suggested multi-strip procedure is very economical: several different proteins may be analyzed from one load of precious sample in one immunoblotting cycle. Furthermore, our modification permits examining up to ten different proteins from one gel lane and transfer up to one hundred different or repeated samples onto a single membrane (Fig. 2), in addition dramatically saving costly antibodies. If gel systems with more wells were used, the data output would increase proportionally. For example, a multi-strip procedure using 15-well gels would generate a membrane holding up to 150 bands, originated from either unique or replicate samples.

Statistical analysis of data is required to differentiate between functionally significant effects and differences caused by sampling variation. For reliability, at least three independent experiments should be performed, where all experimental data points must be run at least in triplicate. Inhomogeneity of the gel, which generally depends on its age and size, can severely impair the quality of replicates, increasing data variability. The use of smaller fresh gels reduces errors caused by gel inhomogeneity, while multi-strip transfer dramatically increases the number of simultaneously detectable bands. Therefore our approach is suitable for generating multiplicates with highly reproducible signal, which does not depend on the lane position within the gel (Fig. 3A) or amplitude of the signal (Fig. 3B).

3.4 Multi-strip Western blotting reduces transfer-derived error

We tested if protein transfer onto the same membrane under equal transfer conditions (the same pH, methanol/Tris-Gly/SDS concentration, transfer time, strength of the contact between gel and the membrane etc.) attenuates transfer-based signal variability (Fig. 4). To compare the standard deviations of signal intensities, decreasing concentrations of recombinant protein (Fig. 4A) or proteins of various molecular weights from fixed-concentration samples (Fig. 4B) were transferred i) onto three separate gel-sized membranes or ii) a single membrane by means of multi-strip modified procedure.

The results indicate that the relationship between the chemiluminescence signal and the quantity of recombinant protein is sigmoidal. The detection limit for recombinant protein was 150 pg per lane in our system. The signal became non-linear above 35 ng of protein per lane. The variability was more pronounced at low rather than high signal intensity, and multi-strip procedure was especially favorable for the improvement of faint signal quality from 2.5 to 4.3 fold (Fig. 4A).

On average, the standard deviation of signal intensities, detected after multi-strip Western blotting of various molecular weight proteins, decreased two-fold in comparison with the

protein transfer onto separate membranes (e.g. 1.68 fold for activated EGFR, 2.03 fold for tyrosine-phosphorylated GAB1, 2.12 fold for regulatory subunit p85 of PI3K, 3.40 fold for tyrosine phosphorylated SHP2 phosphatase, 1.78 fold for phosphorylated ERK1/2 and 1.81 fold for adaptor protein Grb2 (Fig. 4B).

The large surface of gel-sized membranes is often unequally covered with immunoblotting reagents, increasing uneven background. When whole membrane is immunoblotted for a single protein, poor antibodies may additionally cause the occurrence of non-specific bands that interfere or overshadow weaker specific signals (Fig. 3S in the Supplement). Cutting a gel strip with a protein of interest in the middle helps to concentrate antibodies on the more narrow area of the membrane in subsequent incubation steps after protein transfer, and to diminish these drawbacks.

3.5 Application of multi-strip Western blotting for comparative analysis

The best comparative quantitative analysis could be achieved, when the samples are loaded onto the same gel, because each electrophoretic and following immunoblotting cycles are performed under slightly different conditions (which increases data variability and thus restricts comparative quantitative analysis of bands that are visualized on different blots). But when the number of samples exceeds the number of lanes of one gel and multiple data points from one or several experiments should be plotted and compared in a single graph, our modified Western blotting procedure becomes a necessity.

Fig. 5A illustrates an experiment that was designed to compare the activation kinetics of phosphorylated ERK protein from zone ⑤ (left panel) under three different stimulation conditions and in the presence of one perturbation. The cells were treated with increasing concentrations of EGF for various periods of time in the presence or absence of the general PKC inhibitor Bisindolylmaleimide I. The use of multi-strip Western procedure eliminated the need to strip the membrane or load another set of sample just to detect the signal of some house-keeping protein or non-phosphorylated ERK. Zone ⑥ protein GRB2 (right panel), which was obtained from the same gels and transferred onto another single membrane, served as corresponding loading control and was used to quantify the relative signal of phosphorylated ERK. Consequently, the time-course curves of activated ERK could be compared in a single graph (not shown).

Multi-strip Western blotting facilitates the detection of a large number of successive time-course data points without losing quality of the signal. With the help of this procedure, the time-course of protein activation in response to a specific stimulus may be markedly extended to include up to one hundred unique or repeated time-points (Fig. 2). Up to fifty data points in a time-course may be obtained if the experiment is designed to test an effect of a single perturbation, up to thirty three or twenty five – for two or three perturbations, respectively, in each experiment.

Fig. 5B represents an experiment, where the detailed time-courses of EGF-induced ERK activation had to be measured and compared in the absence or presence of PI3K inhibitor wortmannin (total – 24 data points). The comparison is more reliable, when a post-perturbation sample lies next to the control sample in a gel. To compare all 24 samples by conventional procedure, we would have to partition the sequence of data points, load them separately onto three mini-gels (8 samples per one gel) and transfer onto three different membranes. However, the data obtained from these membranes, could not be plotted in a seamless time-course graph. On the contrary, multi-strip Western blotting procedure allowed the reassembling of all data points onto one membrane for simultaneous exposure. Several proteins were detected after just one transfer cycle: phosphorylated ERK from zone ⑤ (Fig. 5B - left panel), 60kDa

phosphorylated AKT, a substrate of PI3K and a suitable readout of PI3K activity, from zone ④, and 70 kDa tyrosine phosphatase SHP2, used as loading control (Fig. 5B - right panel).

There are situations, where it is impossible to separate some proteins in gels due to the close migration of these proteins in the same narrow zone. In this case, we use the strategy of simultaneous detection: a single strip, containing both proteins, is cut out of each gel and transferred onto the same membrane, which is later incubated in a mixture of primary antibodies (Fig. 5B – right panel).

The time interval between independent experiments may be very long. In most cases, data from one experiment needs to be analyzed prior to designing the next experiment, which may be performed under different conditions and then compared with the former. In this case, it is problematic to integrate independently measured data. We suggest a novel multi-strip-based approach for the integration of distinct data sets (Fig. 6)

Fig. 6A is an example of multi-strip Western blotting application for the simultaneous (synchronous) comparison of data from several independent experiments. In this case, the cell lysates after each previous experiment are simply frozen and stored until all the lysates are prepared. At that time, all samples are prepared in Laemmli buffer and processed together.

The principle of separate (asynchronous) comparison of the data from several independent experiments is shown on the Fig. 6B. In the preliminary experiments, a set of different dilutions of a chosen recombinant protein should be selected to provide a linear range of signal intensities for the given conditions (including antibodies, substrate, exposure time, detection instrument etc.) and the detection method of choice. These dilutions should be used throughout all measurements.

A gel strip, containing the set of chosen dilutions, is combined onto an assembling filter and transferred onto the same membrane together with other gel strips that contain samples under investigation. Post detection, the signal intensities of recombinant protein bands are used to generate a standard calibration curve. Thus separate protein detection will result in calibration curve with individual slope. Upper right panel in Fig. 6B compares calibration curves from two independent experiments. Individual calibration curves are used to convert raw signal intensities of appropriate data (lower left panel) into the standardized protein amounts (ng of protein-of-interest per μg of total protein in lysate) (lower right panel). The use of the same dilutions of recombinant proteins eliminates the variation in immunoblotting conditions and instrumental detection from one experiment to another and provides an absolute scale for more accurate comparison of data from two or more different experiments.

4 Concluding Remarks

With multi-strip Western blotting the experimental design becomes more flexible. A single membrane, containing strips from multiple gels, can reveal changes in protein activation and/or concentration upon multiple input factors: type and concentration of ligands, duration of the stimulus, presence of inhibitors or activators, blocking peptides, changes in expression levels of endogenous protein etc. The modification of Western blotting procedure described here meets the requirements of rapid, economical and productive data processing, which maintains high sensitivity and improves the accuracy of immunoblotting, which is critical in proteomics and protein biochemistry studies.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

AKT	protein kinase B
EGF	epidermal growth factor
EGFR	epidermal growth factor receptor
ERK	extracellular-signal-regulated kinase
GAB1	GRB2-associated binding protein 1
GRB2	growth factor receptor-bound protein 2
HGF	hepatocyte growth factor
IGF-1	insulin-like growth factor 1
LDS	lithium dodecyl sulphate
MAPK	mitogen-activated protein kinase
PI3K	phosphoinositide-3 kinase
RasGAP	Ras GTPase activating protein
SHP2	tyrosine-protein phosphatase non-receptor type 11

Fig. 1. Schematic representation of multi-strip Western blotting procedure

A – sample loading and separation by electrophoresis step. B – The measurement of protein molecular weight marker (M) migration relative to the dye line (H) and identification of protein migration zones (shown as numbers in circle). Representative protein within each migration zone is depicted as a set of horizontal bands (dashed line). Scissor symbols indicate the cutting lines, which would separate the entire gel into eight strips. C-F – The strips are assembled onto the filter paper side by side and parallel to each other in different combinations and covered with the nitrocellulose membrane for the subsequent electroblotting.

Fig. 2. Multi-strip Western blotting procedure increases data output ten fold per one immunoblotting cycle

A549 cells were cultured, starved, left untreated or stimulated with 1 nM EGF for 5 minutes, and lysed as described under section “Materials and Methods”. Equal volumes of non-stimulated-cell lysate was loaded onto odd wells (1, 3, 5, 7, 9), while stimulated-cell lysate was loaded onto even lanes (2, 4, 6, 8, 10) of ten (A through J) Novex 4-12% gradient Bis-Tris 10-well mini-gels. Proteins were resolved by LDS-PAGE under reducing conditions. Each gel was divided into six strips containing proteins from zone ① to ⑦, according to the Precision Plus Protein standards marker migration statistics from Table 1. The same protein zone strips from gels A to J (indicated by hand symbols) were combined onto appropriate assembling filter

papers. The proteins from each assembling filter were respectively transferred onto nitrocellulose membrane and immunoblotted for various proteins. Representative blots with ten replicates of phosphorylated (p) p-SHP2 (Tyr542) (**upper panel** - zone ④), p-ERK (**middle panel** - zone ⑤) and GRB2 (**bottom panel** - zone ⑥) are shown. Other detected proteins were p-EGFR (Tyr1173) (zone ①), p-GAB1 (Tyr627) (zone ② ③) and Ras (zone ⑦) (not shown).

Fig. 3. Increased reproducibility of both high and low signals in multi-strip Western blotting

A. Two different dilutions (100% and 50%) of HEK293 cell lysate were prepared and loaded onto three (A, B, C) Novex 4-12% gradient Bis-Tris 10-well gels. Equal volumes of 100% lysate was loaded onto odd wells of gels A and C (lanes 1, 3, 5, 7) and onto even wells of gel B (lanes 2, 4, 6, 8). 50% lysate was loaded onto the rest of the wells. Proteins were separated by LDS-PAGE. Zone ② strip with proteins of molecular weight from 100 kDa to 150 kDa was cut from each gel according to the migration of 10 μ l Precision Plus Protein standards marker (M). All three strips were combined on one assembling filter paper. The proteins were transferred onto the same nitrocellulose membrane and immunoblotted (IB) for RasGAP protein. **B.** Starved HEK293 cells were stimulated with 1 nM EGF for indicated time periods and lysed. Identical sample sets were loaded onto four Novex 4-12% gradient Bis-Tris 10-well gels (A-D) for LDS-PAGE. Zone ④ strips were cut out, according to the migration of 10 μ l Precision Plus Protein standards marker statistics from Table 1, combined onto assembling filter paper, covered with nitrocellulose membrane and electroblotted. The BSA-blocked membrane was cut into two pieces. The first piece was immunoblotted (IB) for p-SHP2, the second – for SHP2.

Fig. 4. Signal variability is markedly reduced after multi-strip Western blotting procedure

A. Serial dilutions (1:1.5) of active recombinant MAP kinase protein (aERK) were used to generate the calibration curve derived from immunoblotting of protein samples either on separate membranes (“**Separate transfer**” - solid line) or on one membrane (“**Multi-strip transfer**” – dashed line). **Left panel** shows the whole linear and non-linear dynamic range of the chemiluminescent signal. Zoom of low-end signal is displayed in the **right panel**. Error bars represent the standard deviation of the mean of triplicate measurements. **B.** MCF-10A cells were cultured, starved, stimulated with 10 nM EGF for 10 minutes, and lysates were prepared as described under section “Materials and Methods”. Eight replicates of the same sample were loaded on each of the six Novex 4-12% gradient Bis-Tris 10-well mini-gels (gel 1 through gel 6). Proteins were separated by LDS-PAGE under reducing conditions. Gels 1, 2 and 3 were covered with nitrocellulose membranes and subjected to protein transfer in separate blot modules. The membranes were cut into six pieces that were separately immunoblotted for phospho-EGFR, phospho-GAB1, PI3K-p85, phospho-SHP2, phospho-ERK1/2 and GRB2 proteins. Each of these proteins was detected by exposing the appropriate membrane pieces

from gels 1, 2, 3 simultaneously (“**Separate transfer**” bars). Alternatively, gels 4, 5 and 6 were used in multi-strip blotting procedure. For detection of each selected protein listed above, three corresponding gel strips were subjected to transfer onto single membrane (“**Multi-strip transfer**” bars) and immunoblotted. Bars represent the mean of signal intensities of twenty four bands (eight sample replicates from three gels) with error bars corresponding to standard deviation of the mean.

Fig. 5.

A. HEK293 cells were starved overnight, and prior to stimulation incubated with 1 μ M of Bisindolylmaleimide I or DMSO for 1 hour. Inhibitor untreated cells were incubated with DMSO (gel A), 0.1 nM (gel E), 1 nM (gel F) or 10 nM (gel G) of EGF for 1.5, 3, 5, 7.5, 10, 15, 30 minutes or left unstimulated (0 min). Inhibitor-treated cells were stimulated with 0.1 nM (gel B), 1 nM (gel C) or 10 nM (gel D) of EGF for the same periods of time of left unstimulated (0 min). Equal volumes of appropriate set of samples were loaded onto Novex 4-12% gradient Bis-Tris 10-well mini-gels A through G, and separated by LDS-PAGE under reducing conditions. Zone ⑤ (**right panel**), zone ⑥ (**left panel**) and other zone (not shown) strips were cut out of each gel, according to the migration of 10 μ l Precision Plus Protein standards marker (M). The same zone strips from each gel were combined onto assembling filter paper, covered with nitrocellulose membrane and electroblotted. The membrane with transferred zone ⑤ proteins was immunoblotted for phospho-ERK (**left panel**). The membrane with zone ⑥ proteins was immunoblotted for GRB2 (loading control) (**right panel**). **B.** HEK293 cells were starved overnight, treated with DMSO (W-) or 100 nM of wortmannin (W+) for 20 minutes, stimulated with 1 nM EGF for 0.5, 1, 2, 3, 4, 5, 7.5, 10, 15, 20, 30 minutes or left unstimulated (0 min), and lysed as described under section "Materials and Methods". The sample pairs of time points from 0 to 2 min were directly loaded onto Novex 4-12% gradient Bis-Tris 10-well mini-gel A, from 3 to 7.5 min – onto gel B, and from 10 to 30 min – onto gel C. Proteins were resolved by LDS-PAGE under reducing conditions. Zone ④ and zone ⑤ strips were cut out of each gel, according to the migration of 10 μ l Precision Plus Protein standards marker (M), assembled onto filter paper, covered with one nitrocellulose membrane and electroblotted. After transfer, the BSA-blocked membrane was cut into two pieces. The piece containing zone ④ proteins was immunoblotted for both phospho-AKT and phospho-SHP2 using the mixture of respective polyclonal antibodies (**right panel**). The piece with transferred zone ⑤ proteins was immunoblotted for phospho-ERK1/2 (**left panel**).

Fig. 6. Multi-strip Western blotting serves for both synchronous (A) and asynchronous (B) comparison of independent data sets

A. A549, T24, HEK293 or MCF-10A cells were grown in 60 × 15 mm tissue culture plates until reached 80% confluence. Starved cells were stimulated with 0.1, 1 and 10 nM of EGF for 5 minutes or left untreated (control) and lysed. Gel A was loaded with straight A549 and reversed T24 sample sets. Gel B was similarly loaded with sample sets prepared from HEK293 and MCF-10A cells. The multi-strip immunoblotting procedure was performed as described above to visualize p-AKT and α-Tubulin (loading control). **B.** Starved T24 or HEK293 cells were stimulated with indicated concentrations (nM) of IGF-1, EGF and HGF for 5 minutes and lysed in two separate experiments. In each case, the proteins were separated by LDS-PAGE. For standard curve (STD – **upper right panel**), a total of 20 ng active recombinant MAP kinase protein (aERK2) per first lane was loaded using 20 μl of 1 ng/μl protein. Serial dilutions (1:2) were loaded onto subsequent lanes. The zone ④ gel strip (containing p-ERK) was placed onto assembling filter paper together with zone ⑤ strip (recombinant ERK migrates as 68kDa). Proteins were simultaneously transferred onto single membrane and probed for phospho-ERK (**upper left panel**). **Charts** show the differences of ERK

phosphorylation in response to various stimuli between HEK293 (**black columns**) and T24 (**diagonal striped columns**) cell lines. **Lower left chart** was generated using raw signal intensities from two independent experiments, and **lower right chart** was constructed after conversion of raw signal intensities into absolute protein amounts using the coefficient, derived from standard calibration curves from separate experiments (**upper right panel**). Absolute protein amounts were divided by appropriate total protein amounts, measured as described in “Materials and Methods” section.

Table 1

Migration of various signal transduction proteins in Novex 4-12% gradient NuPAGE mini-gel and their cutting areas, based on the migration of prestained Precision Plus Protein marker (BIO-RAD).

Zone	Protein MW (kDa)	Cut area (mm from the dye line, H)	Migration range of signal transduction proteins in the gel			
1	150 – 250	49 ± 1 – 54 ± 1	EGFR, ErbB3, PDGFR, CSFR	c-Met, PLCs, FGFR, c-Kit, SHIP, JAK/Tyk2	SOS, IRS1/4, Filamin	VEGFR, c-Ret, ASK1, RhoGAP
2	100 – 150	44 ± 1 – 49 ± 1	RasGAP, FAK, c-Cbl, Cas, PLD1, PTP α		PI3K-p110, GAB1, Gab2, Pyk2, Vav, STATs, MLK3	E-Cadherin, cPLA2, PKD, eNOS, ERK5
3	75 – 100	38 ± 1 – 44 ± 1	PI3K-p85, FRS-2, GRK	IR, PKCs, RSK, IKK α		Raf, Grb10, PAK, p70S6K, FKHR, Calpain
4	50 – 75	29 ± 1 – 38 ± 1	SHP2, Src family, PTEN, Akt, Csk, Grb14	SHC, α -Tubulin, SAPK/JNK, SGK, ILK1, PP2A, CaMKII, GSK3, PTP1B, β -Arrestin, Grb7	Dok-R, Paxillin, p53, PDK1, Sam68, c-Fos	
5	37 – 50	22 ± 1 – 29 ± 1	Crk, ERK, MEK, Nck, CREB		β -Actin, PKA, MAPKAPK2, c-Jun, MKKs	Sprouty, p38 MAPK, GAPDH
6	25 – 37	12 ± 1 – 22 ± 1	I4-3-3, Bic	Bcl-2, PPI, SgRP		
7	20 – 25	9 ± 1 – 12 ± 1	Bad, Rac1/cdc42		Bak, GRB2	
8	15 – 20	5 – 9 ± 1	Survivin, Bmf	Caveolin-1, Ras, DAPI, RKIP		
9	10 – 15	2 – 5			TCL1	BID