Chapter 23

Multistrip Western Blotting: A Tool for Comparative Quantitative Analysis of Multiple Proteins

Edita Aksamitiene, Jan B. Hoek, and Anatoly Kiyatkin

Abstract

The qualitative and quantitative measurements of protein abundance and modification states are essential in understanding their functions in diverse cellular processes. Typical Western blotting, though sensitive, is prone to produce substantial errors and is not readily adapted to high-throughput technologies. Multistrip Western blotting is a modified immunoblotting procedure based on simultaneous electrophoretic transfer of proteins from multiple strips of polyacrylamide gels to a single membrane sheet. In comparison with the conventional technique, Multistrip Western blotting increases data output per single blotting cycle up to tenfold; allows concurrent measurement of up to nine different total and/or posttranslationally modified protein expression obtained from the same loading of the sample; and substantially improves the data accuracy by reducing immunoblotting-derived signal errors. This approach enables statistically reliable comparison of different or repeated sets of data and therefore is advantageous to apply in biomedical diagnostics, systems biology, and cell signaling research.

Key words Western blotting, Electrophoretic transfer, Gel cutting, Quantitative protein analysis, High-throughput, Blotting errors, Systems biology, Cell signaling

1 Introduction

Qualitative measurement of protein abundance is one of the common tasks in biomedical diagnostics in the search for therapeutic targets and biomarkers of various diseases and disorders [1–8]. The quantitative analysis of protein expression, posttranslational modification states (e.g., phosphorylation), recruitment to specific subcellular compartments, and interaction with other proteins is a paramount goal in systems biology, which explores, predicts, and explains how signaling networks govern cellular behavior by exploiting experimental data-driven mathematical models. To achieve this goal, the cellular response to external stimuli in vivo is often compared to the response obtained under one or more perturbations (e.g., pharmacological inhibitors, drugs, exposure to physiochemical stresses, or the down- or upregulation of

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protein expression). In addition, variations in the concentration of a ligand and/or the time course of stimulation provide deeper insight into the spatiotemporal functioning of a specific cell signaling pathway [9]. Obviously, these tasks require the generation of large amounts of high-quality data points.

Quantitative Western blotting used for the immunodetection and densitometric analysis of relative expression levels of electrophoretically resolved proteins is a sensitive and widely used technique, which, however, has several drawbacks [1, 10]. It is a low-throughput, time-consuming and expensive multistep procedure that often results in images that make comparison of paired experimental samples difficult. Each step of Western blotting (sampling, gel loading, electrophoretic separation and transfer of proteins, immunoblotting, and signal detection) is performed under slightly differing conditions in sequential blotting cycles. This eventually increases data variability, which makes it difficult to compare the signals obtained from different series of biological samples [11]. Therefore the improvements of typical Western blotting procedure are in high demand [12–18].

Multistrip Western blotting (MSWB) is a modified immunoblotting procedure based on simultaneous electrophoretic transfer of proteins from multiple strips of polyacrylamide gels to a single membrane sheet. The proposed modification has several advantages over a classical Western blotting procedure [19].

First, the transfer and the sequential procedures with the blot such as membrane washing, incubation with antibodies, and protein detection are performed under similar conditions. This significantly improves the data accuracy by reducing immunoblotting-derived signal errors.

Second, instead of detection of a single target protein *per* each blotting cycle, many additional proteins of interest that sufficiently differ in their molecular weight (MW) can be simultaneously obtained from a single gel and using same sample load. These proteins can be further visualized by immunoblotting (IB) or by dye staining [20–22]. The analytical power of Western blotting is increased, because a one-step analysis of numerous signaling proteins is more productive, saving time as well as costly materials.

Third, the MSWB approach eliminates the need to reuse single blots by stripping and reprobing, which is known to cause inconsistent and undetermined protein loss from a membrane and therefore remains a subject for improvement [23–25]. Blot reprobing is commonly used for the sequential detection of house-keeping proteins (e.g., actin, tubulin, GAPDH, COX-IV, PBGD, mATPsy6) that are believed to have a stable level of expression across all tissue/cell line samples under various experimental conditions. Therefore, despite the susceptibility to error, these are widely used to serve as internal reference controls for loading normalization, which, in turn, poses distinct challenges [26–35].

Fourth, in MSWB, only specific target protein bands transferred from a narrow area of the gel are synchronously detected on the membrane that is incubated in a single-primary antibody solution. This prevents the antibody cross-reactivity and nonspecific binding problems associated with multiplex detection of different-sized proteins following blot incubation in a mixture of two or more primary antibodies.

Fifth, the gel strip(s) containing serial dilutions of any recombinant protein could be included and used as a calibration curve to quantify an absolute amounts of protein of interest in a sample.

Finally, when the number of samples to be analyzed exceeds the number of wells in a gel, the concurrent quantitative protein analysis can be readily achieved by the MSWB technique, which increases the data output *per* single blotting cycle up to tenfold. As a consequence, a large number of data points can be measured, integrated, and compared on the same graph.

Herein we provide some practical examples of routine signal transduction biological experiments that streamline the strategic planning of how to use the MSWB approach to obtain, visualize, analyze, and represent data graphically in an easy-to-read format. Although the hands-on protocol presented here is developed and optimized for denaturing polyacrylamide gel electrophoresis (PAGE) run in MOPS-SDS buffer under reducing conditions using NuPAGE Novex 4–12 % gradient Bis-Tris mini-gels and XCell™ devices (Life Technologies, Carlsbad, CA), the MSWB is an adaptable technique, which can be used with other gel types and various protein transfer systems and is compatible with any protein electrophoresis methods using discontinuous denaturing buffer systems.

2 Materials

2.1 Preparation of Samples

1. Cell lysis buffer (*see* Table 1 and **Note 1**).
2. 100× protease inhibitor cocktail (*see* Table 2).
3. 100× phosphatase inhibitor cocktail (*see* Table 3).
4. 4× LDS sample buffer, pH 8.5 (*see* Table 4).
5. 10× sample reducing agent (*see* Table 5 and **Note 2**).
6. Dry heated bath.
7. Refrigerated high-speed centrifuge.
8. 1.5–2 mL Eppendorf tubes.
9. Cell scrapers.

2.2 Lithium-Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (LDS-PAGE)

1. Electrophoresis unit: XCell SureLock Mini-Cell units (Life Technologies) (*see* **Note 3**).
2. Gels: Precast 10- or 12-well NuPAGE Novex 4–12 % gradient Bis-Tris Mini-gels, 10×10 cm, 1.0 mm thickness (available from Life Technologies) or 4–12 % gradient ExpressPlus™

Table 1
Suggested composition of self-made 1× lysis buffer for total protein whole-cell lysis (WCL), plasma membrane permeabilization (MP) or protein immunoprecipitation (IP), final concentration (FC) of the compounds in 1× working solution, directions for buffer preparation and storage

	WCL		MP[a]		IP[a]	
Compound	**Amount**	**FC**	**Amount**	**FC**	**Amount**	**FC**
1 M HEPES[b], pH 7.4	2.5 mL	50 mM	2.5 mL	50 mM	1 mL	20 mM
1 M NaCl	7.5 mL	150 mM	7.5 mL	150 mM	7.5 mL	150 mM
50 mM EGTA	1 mL	1 mM	1 mL	1 mM	2.5 mL	2.5 mM
10 % Triton X-100	5 mL	1 %	–		5 mL	1 %
Glycerol[c]	5 mL	10 %	5 mL	10 %	2.5 mL	5 %
Digitonin	–		7.5 mg	150 μg/mL	–	
10 % SDC[d]	2.5 mL	0.5 %	–		–	
10 % SDS	0.5 mL	0.1 %	–		–	
dH_2O	Up to 50 mL		Up to 50 mL		Up to 50 mL	

Store at +4 °C. Prior to lysis, supplement the buffer with phosphatase and protease inhibitors. Keep on ice
[a]Details of cell fractionation into cytosolic and particulate fractions and immunoprecipitation are described elsewhere [36]
[b]HEPES interferes with the Lowry, but not the Bradford protein assay and is not suitable for redox studies, because it can form radicals. It may be substituted with Tris–HCl, pH 8.0 or MES, pH 6.8. Be aware that Tris possesses a potentially reactive amine, has high temperature sensitivity, and participates in some enzymatic reactions (e.g., alkaline phosphatase)
[c]Similar to glycerol, 250 mM sucrose also stabilizes integrity of lysosomal membranes, reduces protease release, helps prevent aggregation, and acts as protein cryoprotectant
[d]Sodium deoxycholate may be substituted or supplemented with 70 mM *n*-octyl-β-D-glucoside (add 1 g per 50 mL) to extract low-density Triton X-100-insoluble caveolin-rich membrane domains

Table 2
Suggested composition of self-made 100× protease inhibitor cocktail, final concentration (FC) of enlisted compounds in 1× working solution, directions for preparation, and storage

Compound	Mr (g/mol)	Amount	FC (1×)
AEBSF-HCl	239.69	25 g	104 mM
Aprotinin from bovine lung	6511.44	0.52 g	80 μM
Bestatin-HCl	344.8	1.5 g	4.5 mM
E-64	357.4	0.54 g	1.5 mM
Leupeptin hemisulfate salt	475.6	1 g	2.1 mM
Pepstatin A	685.91	1 g	1.5 mM
DMSO		Up to 10 mL	

Store at −20 °C in 1 mL aliquots. Dilute to 1× in ice-cold lysis buffer immediately prior to lysis

Table 3
Suggested composition of self-made 100× phosphatase inhibitor cocktail, final concentration (FC) of the compounds in 1× solution, target, directions for preparation, and storage

Compound	Mr (g/mol)	Amount	FC (1×)	Target
Sodium fluoride	42	84 mg	2 mM	Acid phosphatases
Imidazole	68.1	136.2 mg	2 mM	Alkaline phosphatases
Sodium molybdate	205.9	236.8 mg	1.15 mM	Acid phosphatases
Sodium tartrate, dihydrate	230.1	920.4 mg	4 mM	Acid phosphatases
Sodium pyrophosphate, decahydrate	416.1	416.1 mg	1 mM	PP1 and PP2A
b-Glycerophosphate	306.1	306.1 mg	1 mM	Ser/Thr phosphatases
200 mM activated sodium orthovanadate solution[a]	183.9	10 mL	2 mM	Tyrosine and alkaline phosphatases

Dissolve the reagents in 200 mM activated sodium orthovanadate solution. Aliquot 1.5 mL of stock cocktail in 2 mL Eppendorf tubes and store at −20 °C (stable at least 6 months). Dilute to 1× in ice-cold lysis buffer immediately before use
[a]Dissolve 3.68 g of Na_3VO_4 in 90 mL dH_2O and adjust to 100 mL. Adjust pH to 10 while stirring. Adding HCl will make the solution yellow. Boil the solution by heating in a microwave for 5–15 s or place it on a heated platform until it becomes clear and colorless. Cool on ice until the solution reaches RT. Add a small amount of 1 M HCl while stirring to adjust pH back to 10. Repeat these steps a total of 3–5 times. After several cycles of boiling, cooling, and pH adjustment, the solution should reach a point of a stable pH at ~10, where adding HCl should result in little, if any, appearance of yellow color. Aliquot and store activated Na_3VO_4 at −20 °C. Stable for at least 6 months

Table 4
The composition of self-made 4× NuPAGE LDS Sample Buffer, final concentration (FC) of the compounds in 4× solution, directions for preparation, and storage

Compound	Mr (g/mol)	Amount	FC (1×)
Tris–HCl[a]	157.56	6.68 g	106 mM
Tris-Base[a]	121.14	6.83 g	141 mM
EDTA disodium salt	372.24	76 mg	0.51 mM
Coomassie G250	854.02	75.15 mg	0.22 mM
Phenol Red	354.38	25.51 mg	0.18 mM
LDS	272.33	4–8 g	1–2 %
Glycerol (≥99 %)	92.09	40 mL	10 %
Ultrapure H_2O		Up to 100 mL	

Mix the reagents well in 30 mL of H_2O, add glycerol, and adjust the volume to 100 mL. pH should be ~8.5. Store at +4–25 °C room temperature (RT) (stable for 6 months)
[a]Tris can be substituted with 0.8 M triethanolamine-Cl, pH 7.6 (FC for 1× is 0.2 M)

Table 5
The composition of self-made 10× Reducing reagent, its final concentration (FC) in 1× solution, directions for preparation and storage

Compound	Mr (g/mol)	Amount	FC (1×)
DL-Dithiothreitol (DTT), anhydrous	154.25	3.85 g	50 mM
dH_2O		Up to 50 mL	

Dissolve in 40 mL of dH_2O. Adjust volume to 50 mL. Store at +4 °C in foil-wrapped or dark centrifuge tubes

Table 6
Recipes for making Bis-Tris resolving gels from 6.0 to 20.0 % and stacking gels of 4 % T

Compound	Resolving gel monomer %						Stacking gel (4 %)
	6 %	8 %	10 %	12 %	15 %	20 %	
4× Bis-Tris–HCl buffer[a], mL	5	5	5	5	5	5	5
30 % T/2.67 % C[b], mL	4	5.3	6.67	8	10	13.3	2.66
dH_2O, mL[c]	10.94	9.64	8.27	6.94	4.94	1.64	12.33
N,N,N′,N′-tetra-methyl-ethylene-diamine (TEMED)[d], µL	5	5	5	5	5	5	10
10 % (w/v) ammonium persulfate (APS)[e, f], µL	50	50	50	50	50	50	100

Total Monomer VOLUME: 20 mL
[a]Dissolve 149.4 g of Bis-Tris in 400 mL of dH_2O and titrate with 37 % HCl until pH reaches 6.5–6.8. Bring volume to 500 mL
[b]Dissolve 0.8 g N′N′-Methylene-Bis-acrylamide and 29.2 g Acrylamide in 70 mL of dH_2O. Bring volume to 100 mL. Filter through a 0.45 µm filter and store at +4 °C in amber glass bottle (30 days maximum). Alternatively, the researcher may use pre-weighted commercially available mixture of 37.5:1 ratio
[c]Adjust water volume if using optional Rhinohide Polyacrylamide Gel Strengthener Concentrate
[d]Use undiluted TEMED
[e]Dissolve 1 g APS in 10 mL of dH_2O. Sterilize by passing through a 0.22 µm filter using 20 mL Luer-Lok syringe. Aliquot to 0.5 mL tubes and store at −20 °C. APS slowly decays in solution, so replace the stock every 2–3 weeks
[f]Degas monomer solution prior to adding catalysts for copolymerization of gels

PAGE Gels with adapters for XCell [available from GenScript (Piscataway, NJ)] (*see* **Note 4**). Alternatively, the researcher may hand-pour the homogenous or gradient Bis-Tris gels using 1.0 mm Gel Cassettes and a gradient former of choice [37]. For instance, homogenous Bis-Tris separating and stacking gels can be prepared from a 30 % T/2.67 % C acrylamide/Bis (37.5:1 ratio) stock solution and a 4× Bis-Tris–HCl stock buffer (Table 6). Be aware that acrylamide and bisacrylamide are carcinogens and neurotoxins when in solution.

3. Prestained molecular weight marker: Precision Plus Protein™ All Blue standards containing a mixture of ten blue-stained recombinant proteins (10–250 kD), including three reference bands (25, 50, and 75 kDa). Store at −20 °C (*see* **Note 5**).

Table 7
The composition of self-made 20× MOPS-SDS Running buffer, final concentration (FC) of the compounds in 1× solution, directions for preparation, and storage

Compound	Mr (g/mol)	Amount	FC (1×)
3-(*N*-morpholino)-propanesulfonic acid (MOPS) (free acid)	209.26	209.26 g	50 mM
Tris (free base)	121.14	121.14 g	50 mM
EDTA, pH 7.7	292.24	5.84 g	1 mM
Sodium-dodecyl sulfate (SDS)	288.372	20 g	0.1 % (w/v)
dH_2O		Up to 1 L	

Mix the reagents well in 800 mL of dH_2O and prior to adding SDS, adjust the pH to 7.7. Adjust the volume to 1 L. Store at RT or at +4 °C (stable for 6 months). For PAGE, dilute this buffer to 1× with dH_2O
Check the pH of this buffer if it is obtained from commercial source other than Life Technologies (#NP0001) or Boston BioProducts (Worcester, MA, #BP-178)

Table 8
The composition of self-made NuPAGE Antioxidant, final concentration (FC) of the compounds in 1× solution, directions for preparation, storage, and commercial source of pre-made reagent (CS)

Compound	Mr (g/mol)	Amount	FC (1×)
Sodium Bisulfite	104.061	7.5 g	15 % (w/w)
N,*N*-Dimethylformamide	73.09	5 mL	10 % (w/w)
dH_2O		Up to 50 mL	

Dissolve sodium bisulfite in 40 mL dH_2O, then under the fume hood VERY SLOWLY add *N*,*N*-Dimethylformamide, and adjust volume to 50 mL with dH_2O. DO NOT SHAKE! Store in sealed dark centrifuge tube at +4 °C. Formation of crystals after some time is acceptable
CS Life Technologies (#NP0005)

4. 20× NuPAGE MOPS-SDS Running buffer (*see* Table 7 and **Note 6**).
5. 1× NuPAGE antioxidant (*see* Table 8).
6. Gel loading tips.

2.3 Western Blotting: Protein Transfer

1. Blotting unit: XCell II™ Blot wet transfer module (*see* **Note 3**).
2. Nitrocellulose membrane, one roll, pore size 0.22 μm (*see* **Note 7**).
3. Filter paper (FP) sheets.
   (a) Extra-thick narrow: 7×10×0.248 cm (W×L×H), 320 grade. For mini-gels cut to 7×9 cm.
   (b) Extra-thick medium: 7.5×10×0.248 cm (W×L×H), 320 grade. For mini-gels cut to 7.5×9 cm.

Table 9
The composition of self-made 1× NuPAGE Transfer buffer, final concentration (FC) of the compounds in 1× solution, directions for preparation, storage, and commercial source of pre-made reagent (CS)

Compound	Mr (g/mol)	Amount	FC (1×)
Bicine	163.17	10.2 g	25 mM
Bis-Tris (free base)	209.24	13.1 g	25 mM
EDTA, pH 7.2	292.24	0.75 g	1 mM
Chlorobutanol (optional preservative)	177.46	0.177 g	0.05 mM
dH_2O		Up to 1 L	

Store at RT. For transfer, dilute 50 mL of 20× buffer with 849 mL dH_2O, add 100 mL Methanol (FC 10 % w/w), and supplement with 1 mL of NuPAGE Antioxidant. The pH of the 1× solution is 7.2. Store at +4 °C
CS Life Technologies (#NP0006-1)

Table 10
The composition of self-made 1× Setup buffer, final concentration (FC) of the compounds in 1× solution, directions for preparation and storage

Compound	Mr (g/mol)	Amount	FC (1×)
Tris (free base)	121.14	12.114 g	25 mM
Glycine	75.066	57.65 g	192 mM
SDS	288.372	4 g	0.1 %
dH_2O	18.01	3,200 mL	
Methanol (add last)	32.04	800 mL	20 % (v/v)

Total VOLUME: 4 L
Buffer pH should be 8.1–8.6, but no adjustment is required. Store at +4 °C

(c) Extra-thick wide: 10 × 15 × 0.248 cm (W × L × H), 320 grade. For mini-gels cut to 8.5 × 9 cm.

(d) Thin: 7.5 × 10 × 0.083 cm (W × L × H), grade 222; For mini-gels cut to 7.5 × 9 cm.

4. A set of firm sponge pads (four pads for one blot). Alternatively, manually cut the 15.2 × 22.8 cm (6″ × 9″) light-duty scour pad into four pieces that fit well in the XCell II Blot module.
5. Gel cutting knife.
6. Flat and upward bent tip tweezers.
7. Gel/Blot assembly trays.
8. Blotting roller, 8.6 cm wide.
9. 20× NuPAGE Transfer buffer (*see* Table 9) and refrigerated dH_2O.
10. 1× setup buffer (*see* Table 10).

Table 11
The composition of self-made 10× TBS-T buffer, final concentration (FC) of the compounds in 1× solution, directions for preparation and storage

Compound	Mr (g/mol)	Amount	FC (1×)
1 M Tris, pH 8.0	121.14	200 mL	10 mM
NaCl	58.44	175.32	150 mM
100 % Triton X-100	647	10 mL	0.5 % (v/v)
dH_2O		Up to 2 L	

Store at RT. For solutions, dilute this buffer to 1× with dH_2O and store at +4 °C

2.4 Western Blotting: Protein Immunodetection

1. Blot incubation dishes: Square Petri Dishes with Grid (*see* **Note 8**).
2. 10× TBS-T buffer: Tris-buffered saline (TBS) with Triton X-100 (*see* Table 11).
3. Blocking buffer: 3 % (w/v) bovine serum albumin (BSA) in 1× TBS-T buffer.
4. Antibody solutions:
   (a) Unconjugated or HRP-linked primary antibodies (1°Ab) of choice diluted in 1× TBS-T.
   (b) Secondary HRP-linked antibodies (2°Ab) of choice diluted in 1× TBS-T.
5. SNAP i.d.® Protein Detection system (EMD Millipore, Upstate, NY) (optional). If used, this system significantly shortens the time of membrane blocking and/or incubation with primary/secondary antibodies. Requires heavily concentrated primary antibody solution.
6. Western blotting detection ECL reagents: SuperSignal West Dura and/or West Pico Extended Duration Chemiluminescent substrates.
7. Titanium Bonded Scissors: 1 for membrane cutting, 1 for filter papers, 1 for opening gel envelopes, 1 for cutting sheet protectors. We strongly advise against using the same scissors for these tasks.
8. 3 M Scotch Magic Transparent Tape and desktop tape dispenser.
9. Imaging system with CCD sensor and zoom for image visualization and densitometric analysis: Image Station 440CF (Eastman Kodak Scientific Imaging Systems, New Haven, CT).
10. Avery® Diamond Clear Heavyweight Quick-Load Sheet Protectors. Each protector can be cut horizontally into three or four pieces, depending on the width of the blot to be protected during its visualization and subsequent storage.

3 Methods

3.1 Preparation of Samples for Multistrip Western Blotting

1. Scrape the stimulated or nonstimulated cells into ice-cold 1× lysis buffer. Suggested volume of buffer used for total protein whole-cell lysis (WCL) of 70–90 % confluent cells grown in 150×15 cm cell tissue culture dishes is 1.6–1.8 mL, in 100×20 cm dishes—1–1.2 mL, for 60×15 cm dishes—0.5–0.6 mL. To homogenize 1 mg of tissue, use 1.5× fold volume of lysis buffer (in mL) (e.g., for 100 mg tissue, add 1.5 mL of lysis buffer). Keep tubes on ice throughout all experiment.
2. Spin the samples at least at 10,000×*g* for 10 min at 4 °C.
3. Mix the supernatant of each cell lysate with 4× NuPAGE LDS sample buffer and 10× NuPAGE sample reducing agent in a ratio of 65:25:10 in prelabeled Eppendorf tubes.
4. Heat samples at 75 °C for 5 min. Cool samples to RT.
5. Run samples within 2 week period. For extended storage, store the samples at 4 °C (reheat prior to loading). Unused amount of cell lysates can be stored for further use at −20 °C/−80 °C.

3.2 Sample Loading and LDS-PAGE

Sample loading strategy highly depends on the design of an optimal experiment to test a hypothesis or answer a biological question. The number of gels to be loaded depends on the number of data series and the number of samples within each series to be analyzed. For example, when the time-course expression of protein of interest in control cells (A) is compared to that under perturbed conditions (e.g., in the presence of inhibitor of protein X (B) and the suppression of protein Y by siRNA (C)) at 0, 1, 3, 5, 7, 10, 20, 30, and 60 min, one will have to load three data series (A, B, and C), consisting of nine time-points each (A1, A2… A9, B1–B9, C1–C9) into three 10-well gels. There are two alternative ways of loading such number of samples (Fig. 1). Further, the loaded samples onto one gel (excluding protein MW marker(s)) will be referred to as a "*set of sample*," sometimes, indicating how many samples are in the set (e.g., one set of nine samples; three sets of eight samples).

1. Carefully remove a comb from each precast gel and rinse its wells and the whole gel under a running stream of dH_2O.
2. Place the first gel in front of the buffer core (FRONT), and the second gel—behind (BACK). In both cases, the shorter (notched) side of the cassette should face in toward the core. Lock the tension wedge.
3. Fill the upper chamber of each XCell SureLock Mini-Cell unit with 200 mL of properly cooled 1× MOPS-SDS Running

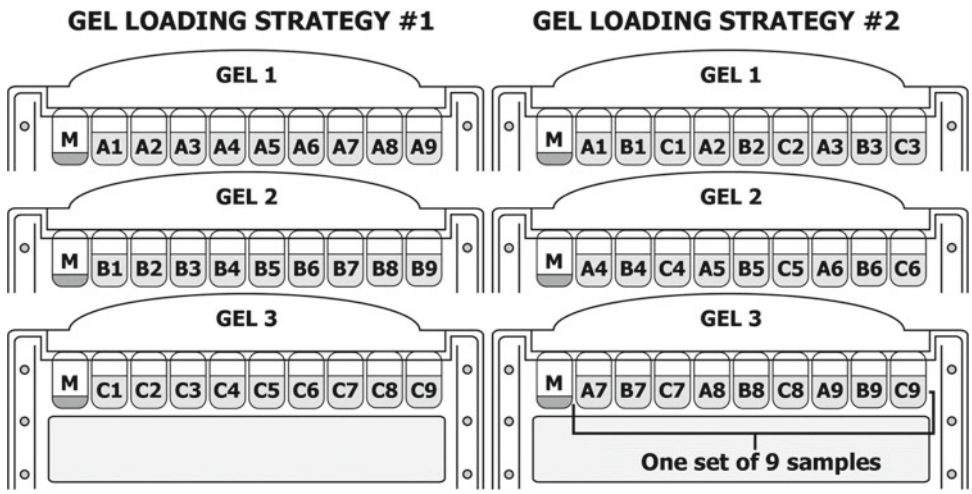

Fig. 1 Gel loading strategies when the number of samples exceeds the number of wells in one gel. M, prestained protein MW marker

buffer to completely cover the sample wells of a gel. Pour ~600 mL of the same buffer into the lower chamber.

4. Prior to sample loading and electrophoresis, supplement the 1× Running buffer in the upper (cathode) chamber of unit with 0.5 mL NuPAGE Antioxidant.
5. Use a pipette equipped with prolonged gel loading tip to underlay 7 μL of prestained protein MW marker into the first and/or the last well of the gel. Loading both wells with a marker can substantially ease and speed up the subsequent step of Gel Cutting (described under Subheading 3.3).
6. Load an equal volume of each sample into the rest of gel wells (*see* **Note 9**). Maximum sample loading volume per well of 1.0 mm 10-well gel is 25–30 μL, 12-well—20–25 μL, 15-well—15 μL. Do not overload! If there are empty wells without loaded sample left, fill them with similar amount of sample buffer diluted in phosphate-buffered saline (PBS buffer, pH 7.4). Overall, load the amount of protein according to the sensitivity of your detection method.
7. Add the lid on the buffer core and connect the apparatus to a power supply. Separate proteins according to their electrophoretic mobility at constant voltage of 125–150 V until the blue dye front (BDF) reaches the bottom of a gel.
8. If running more than two gels, make an interval of at least 5–10 min before loading the next tandem of gels and powering on the electrophoresis unit. This will reserve enough time for follow-up steps. During this pause, mark a sequence of loaded samples in a laboratory notebook along with the details

of the experiment. Also, you may want to attach the sticky notes on the electrophoresis apparatus, helping to identify the gels (e.g., Gel 1—FRONT, Gel 2—BACK).

9. At the end of electrophoresis, remove gel cassette out of apparatus, rinse under a stream of dH_2O and gently open with a gel knife. Note that upon opening the cassette, the gel can be adhered on either side. If the gel remains on a shorter (notched) side of the plate, the sequence of sampling should be rewritten in the laboratory notebook in a reversed order. However, in such case, the strips that will be derived from such reversed gel will inevitably need to be flipped horizontally by imaging software. To avoid possible manipulation, it is advisable to reverse the strips during Assembly step (described under Subheading 3.4).
10. Discard the plate of gel cassette without the gel. Rinse the side with an adhered gel with dH_2O.
11. Proceed to Gel Cutting step as instructed under Subheading 3.3. Preferably, each gel should be cut immediately after opening the cassette as soon as electrophoresis is complete. If you decided to wait until electrophoresis is over and BDF reaches the bottom of ALL gels, then cover adhered gel with extra-thick 7.5 × 8 cm filter paper (further referred as CFP, i.e., covering filter paper), which has been submerged once in cold 1× Setup buffer. Thereafter, put the cassette with covered whole gel aside and stick the label, helping to identify a gel.
12. Perform **steps 9–11** with gel cassettes from other electrophoresis units.

3.3 Gel Cutting

Figure 2 illustrates the plate with attached gel after protein separation according to their molecular weight by LDS-PAGE. The prestained protein MW marker is visibly separated into the bands corresponding to protein molecular weights of 250, 150, 100, 75, 50, 37, 25, 20, 15, and 10 kDa (Fig. 2, *M*). Though this is an optional step, the researcher may firmly position a millimeter-scaled transparent ruler near the edge lane with separated marker so that zero (0 cm) aligns with the middle of the *BDF* (Fig. 2). The distance from the *BDF* to the center of each marker band (Fig. 2, *H*) is measured in millimeters and can be registered in a statistical table (*see* **Note 10** and Table 12).

The distance between two electrophoretically separated marker bands corresponds to the migration range of certain molecular weight proteins. For instance, the distance between *H250* and *H150* defines a migration range of electrophoretically separated proteins with molecular sizes between 150 and 250 kDa. This area is termed zone ①. Each sample provides up to nine protein-containing zones that may be simultaneously cut out from a single gel (Fig. 2).

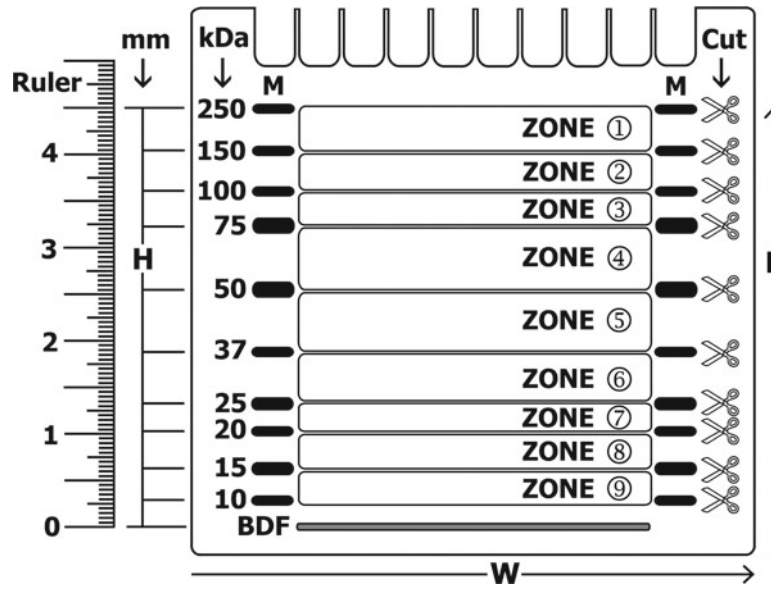

Fig. 2 Identification of protein migration zones in Multistrip Western blotting procedure. First and last lanes show separated prestained protein MW marker (*M*). *BDF* indicates blue dye front; *H* is the distance from *BDF* to the center of particular marker band. *Scissors symbols* indicate the cutting lines, which would separate the entire gel into nine strips. Protein migration zones are enumerated by *numbers in circles. L*—gel plate length, *W*—gel cassette width

The number of strips to be cut out from the gel depends on the number of distinct proteins to be analyzed. Most frequently studied signal transduction proteins migrating in various zones are listed in Table 13, which also indicates the appropriate areas that can be cut out of the gel for subsequent detection of these proteins. These areas may be narrow (containing only one Zone) or wide (spanning two or more Zones).

For example, Fig. 3 depicts the strategy of gel cutting into strips by the researcher who seeks to examine changes in the levels of multiple biochemical markers of programmed cell death (apoptosis). Only four blotting cycles (or eight if two gels are used) allow the researcher to separate and detect a group of distinct apoptosis- and cell survival-related proteins whose migration in a gel often overlap. Importantly, if the researcher wants to detect only those proteins enlisted in the *left lower panel of* Fig. 3 and has loaded only one set of eight samples for concurrent measurement and comparison of signals, there is no need to cut a gel into strips. He/she should proceed to the Assembly of Western blotting sandwich step (described under Subheading 3.5), transfer the proteins onto the membrane from the entire uncut gel, and thereafter (using clean Titanium scissors) cut the membrane into four strips containing Zone ①②, Zone ③④, Zone ④⑤, and Zone ⑤⑥⑦⑧⑨, respectively.

Table 12
The example of a template of Statistical Table to record the migration patterns of various prestained molecular standards (markers) in various types of gels

Protein MW standards (M) title, commercial source	Running Buffer	Zone	MW bands (kDa)	Cut area (H) (distance in mm from BDF) in various gels		
				10% Bis-Tris	4-12% Bis-Tris	4-20% Tris-Glycine
BlueRay Prestained Protein Marker (10-180 kDa) Jena Bioscience #PS-103	MES-SDS	1	125-165			
		2	93-125			
		3	72-93			
		4	57-72			
		5	42-57			
		6	31-42			
		7	24-31			
		8	15-24			
		9	8-24			
Precision Plus Protein Dual Xtra Standards (2-250 kDa) Bio-Rad #161-0377	MOPS-SDS	1	150-250		49 to 55	
		2	100-150		44 to 49	
		3	75-100		38 to 44	
		4	50-75		29 to 38	
		5	37-50		22 to 29	
		6	25-37		12 to 22	
		7	20-25		9 to 12	
		8	15-20		5 to 9	
		9	10-15		2 to 5	
		10	5-10		0 to 2	
		11	2-5		BDF	

If you need to compare several sets of samples that were resolved in *two* or *more* gels, then proceed to Subheading 3.3, **step 1**.

1. Starting from the bottom and moving toward the top of the adhered gel, use a regular gel knife (or blade) to cut out the strip, which covers an area with a protein of interest located in the middle, from the gel across its entire width, including the lane(s) with a separated protein MW marker (Figs. 2 and 3, *scissors symbol*).
2. Proceed to cutting the next strip above.
3. Discard the gel pieces outside the strips.
4. Cover the first plate with prepared multiple gel strips with a sheet of moistened *CFP* and place on the bench top. Similarly, cut the second gel, cover it with another sheet of CFP, and place it next to the previously laid plate. Repeat above procedure with the rest of the gels.

Table 13
Migration range of signal transduction proteins in NuPAGE 4–12 % gradient mini-gel and their cutting areas, based on the migration pattern of prestained Precision Plus All Blue Protein marker

Zone	Protein MW range (kDa)	Cut area, H (mm from BDF)	Suggested cutting guidelines of gradient 4-12% gel strips for subsequent detection of indicated signal transduction proteins				
1	150 – 250	from 49 ± 1 to 54 ± 1	EGFR, ErbBs, MRP2, PDGFR, CSFR, Rictor	c-Met, PLCs, FGFR, c-Kit, SHIP, Tyk2, MDR1, Raptor, Eps15, ROCK1, ASK1, Collagen I, eNOS	SOS, IRS1/2/3/4, ZEB1, Filamin, Alk	VEGFR, c-Ret, BRCA1, Fibronectin	Bcr-Abl, RhoGAP
2	100 – 150	from 44 ± 1 to 49 ± 1	RasGAP, FAK, c-Cbl, SirT-1, PLD1, PTPα, Pecam-1, Vinculin, JAK1/2/3		PI3K-p110, Gab1/2, Pyk2, Vav, MLK3, β-Catenin, STATs, Fer, Rb, p105 NF-κB1, VE-Cadherin, FoxO3a, PARP (FL and CL)	E/N-Cadherins, PKD, cPLA2, c-Abl, gp130, ERK5, HIF-1α, MCM2, p130Cas	MMP-9, PLD2, Exportin, TRIF, Integrin β1
3	75 – 100	from 38 ± 1 to 44 ± 1	PI3K-p85, FRS-2, GRK, APS, HIF-1β, Wee1, Eps8	IGF-1R, InsR, PKCs, p90RSK, IKKα, Hsp90, FoxO1		Raf family, MXR, Grb10, FKHR, p70 S6K, Calpain, Gab3, Egr1, MMP-2, Lamins A/B/C, PAK 4/5/6, cdc25A/B/C	
4	50 – 75	from 29 ± 1 to 38 ± 1	SHP1/2, c-Src family, PTEN, Csk, Akt 1/2/3, AFP, Grb14, Grb7, Myc, PAK1/2/3, CDT1, RIP3, MT-MMP-1	α-Tubulin, Shc, p53, JNK1/2, SGK, ILK1, GATA-2/3, PP2A, PAI-1, CaMKII, GSK-3 α/β, PTP1B, β-Arrestin, IκBβ, Caspase-9 (FL), c-Fos, Cyclin E, Vimentin, MMP-1/3, p50 NF-κB1, Keratin 8/17/18	Dok-R, Paxillin, PDK1, Sam68, SRF, Chk1/2, AMPKα/γ, STAM1, Hsp70, Myt1, Cyclin A, Cyclin B, RPA1		FoxO4, WASP, AIF, ZAP-70, Syk, GATA-6, LIMK-1/2, p65 NF-κB1, Merlin
5	37 – 50	from 22 ± 1 to 29 ± 1	Crk, ERK1/2, MEK, Nck, CREB, AMPKβ, FRA1, GATA-1, PAR-4		β-Actin, PKA, MKKs, c-Jun, Flotillin-1/2, SMA MAPKAPK2	Sprouty, PCNA, CDK6, LAT, GAPDH, siRT-2, p38 MAPK, Caspase-3/7 (FL), Caspase-9 (CL), Cyclin D, Pim-1/2/3	TGF-β1/2/3, VEGF isoforms, TNF-α, TGF-α IL-6, IL-10, IL-1 α/β, PDGF-A/B, HB-EGF (all mature and precursor) MMP-7
6	25 - 37	from 12 ± 1 to 22 ± 1	14-3-3, Bic, VDAC, Slug, Snail, Twist	Bcl-2, PP1, S6RP, HO-1, cdc2, CDK2, CDK4	Bak, GRB2, Bcl-xL, Hsp27, p27 Kip1, Lamin A (CL small subunit)		
7	20 - 25	from 9 ± 1 to 12 ± 1	Bad, Rac1/cdc42, Puma	Bax, Caveolin-1, p21, Ras, DAP1, RKIP, Claudin-1, Cofilin		BID, MCP1, IGF-1, Caspase-3/7/9 (CL)	
8	15 - 20	from 5 to 9 ± 1	Survivin, Bmf, p18, COX IV		TCL1, Cytochrome c, Angiogenin 1, Histone H3/H2A.X		
9	10 - 15	from 2 to 5					

3.4 Assembly of Gel Strips

During this step, the gel strips that are derived from different gels are assembled onto a single sheet of filter paper (*AFP*, for assembling filter paper) for the subsequent electrophoretic protein transfer onto the same piece of nitrocellulose membrane (*see* **Note 7**). The strategy of assembly depends on the quantity of gels used for PAGE as well on the number of strips containing the appropriate proteins of interest (*see* **Note 11**).

Here we provide two exemplar cases of gel strip assembly:

(i) If one loaded five sets of samples in five gels (GEL1–5) and subsequently cut five strips to detect five proteins of interest (PARP, p-Chk1, β-Actin, Bcl-2, and Cytochrome c) as shown in *left upper panel of* Fig. 3.

(ii) If one loaded three sets of samples in three gels (GEL1, GEL2, and GEL3) and subsequently cut five strips to detect five proteins of interest (p-BRCA1, E-Cadherin, c-Myc, p-c-Jun, and Bax) as shown in *right lower panel of* Fig. 3.

1. In case (i), flip and gently lift the plate containing GEL1 so that all strips would stick to the moistened CFP. Use gel knife if the strips do not independently detach from the plate.

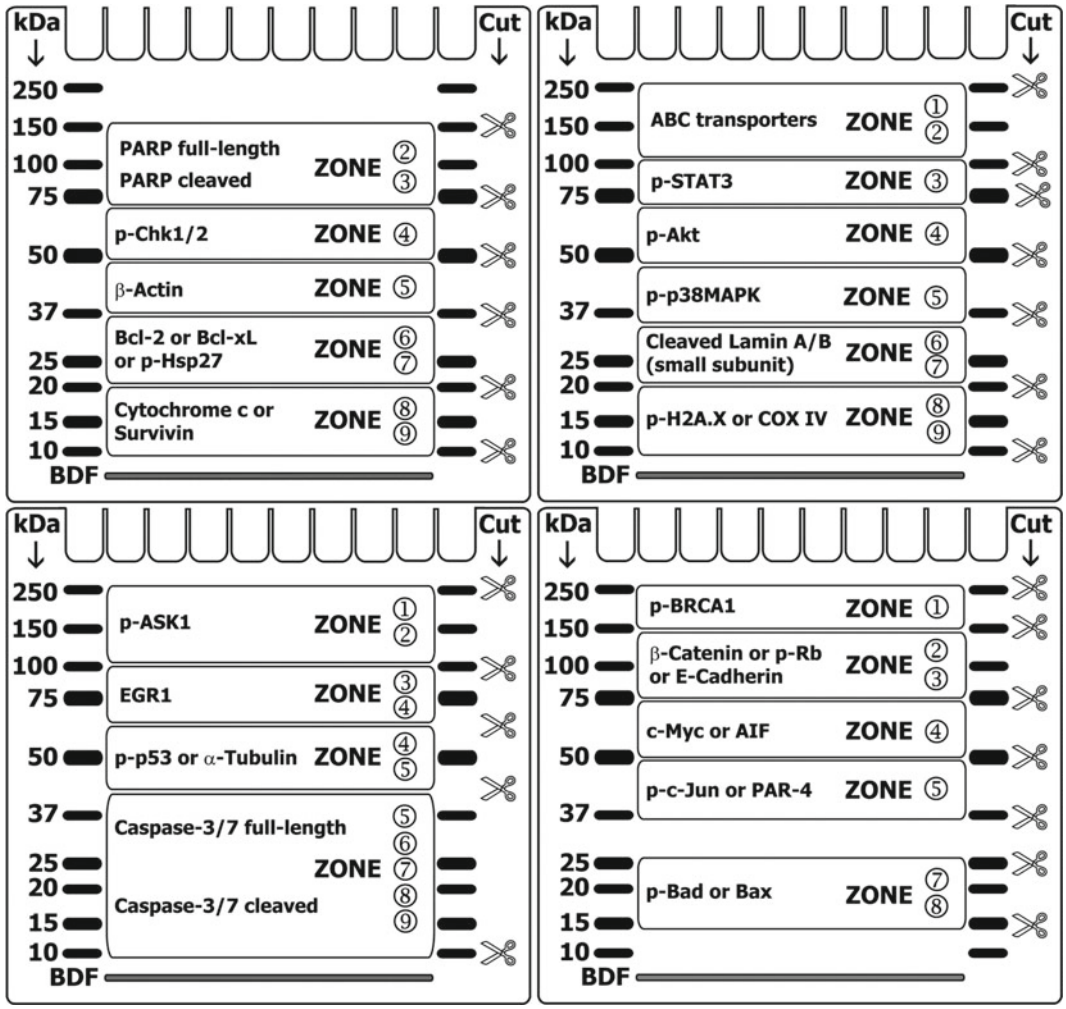

Fig. 3 Suggested gel cutting for analysis of proteins implicated in apoptosis and cell survival

2. Lift the first gel strip from the top (possessing Zone ②③ proteins) with a gloved hand and carefully transfer onto AFP #1 that must be briefly presoaked in 1× Setup buffer. Return the CFP with remaining gel strips onto the plate by flipping it back.
3. Similarly, top gel strips derived from GEL2–GEL4 and GEL5 are sequentially transferred onto the AFP #1 so that the strips would lay side by side and parallel to each other. AFP #1 is now ready for immediate protein transfer (*see* **Note 12**).
4. **Steps 1–3** are repeated with the strips derived from GEL1 to GEL5 that contain the proteins migrating in Zone ④, Zone ⑤,

then Zone ⑥⑦, and finally in Zone ⑧⑨. This procedure will yield five AFPs (AFP #1–#5) with collected five gel strips on each (Fig. 4). Now they are ready for assembly of Western Blotting sandwich and subsequent electrophoretic protein transfer onto the same nitrocellulose membrane.

5. In case (ii), perform **steps 1–2** with GEL1, followed by a sequential transfer of gel strips derived from GEL2 and GEL3 onto the AFP #1. Then, place gel strips with Zone ②③ onto the AFP #1 below previously laid triplet of strips. Leave a small gap between the triplets (Fig. 5, *upper panel*).
6. AFP #2 is processed in the same manner so that it would contain triplet of strips with Zone ④ and triplet of strips with Zone ⑤ (Fig. 5, *middle panel*). After protein transfer, the resulting membrane is cut into two pieces across the gap between the triplets (Fig. 5, *scissors symbol*). The pieces are then treated in separate dishes (*see* **Notes 8** and **13**).
7. The remaining three strips containing Zone ⑦⑧ should be assembled onto AFP #3 (Fig. 5, *lower panel*).

3.5 Assembly of Western Blotting Sandwich and Protein Transfer

Instructions provided below assume the use of XCell II Blot module, which is used for transfer of protein from one AFP.

1. Fill one side of gel/blot assembly tray with 500 mL of refrigerated 1× Setup buffer, while another side—with 400 mL of 1× Transfer buffer.
2. Presoak four sponge pads in 1× Setup buffer. Remove air bubbles by squeezing the pads while they are submerged in buffer. Cut a sheet of nitrocellulose membrane to the dimensions of AFP and presoak it in Transfer buffer for 5 min before using. Briefly moisten three additional extra-thick and one thin filter papers in Setup buffer immediately before using.
3. Place two wet sponge pads into the cathode (−) core of the blot module. Place the AFP with collected gel strips on the top. Subsequently, cover the surface of gel strips with a sheet of membrane. Remove any trapped air bubbles by rolling a blotting roller over the membrane surface. Place three moistened extra-thick filters onto the surface of the membrane followed by tandem of wet sponge pads (*see* **Note 14**).
4. Place the anode (+) core on the top of the pads. Slide the blot module into the rails on the lower chamber. Lock the gel tension lever.
5. Fill the blot module with 1× Transfer buffer until the blotting sandwich is completely submerged. Fill the outer chamber with chilled dH_2O.

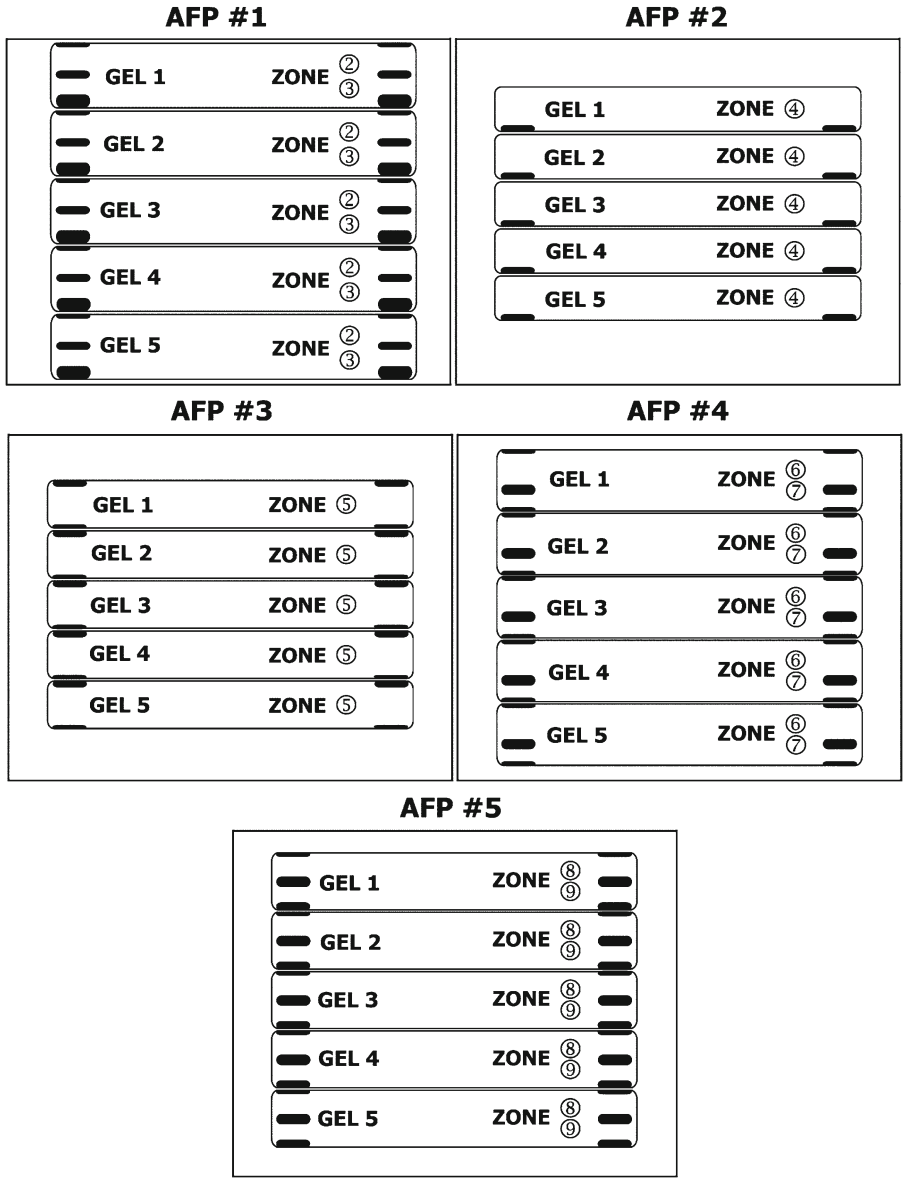

Fig. 4 A strategy for assembly of five gel strips following LDS-PAGE of five sets of samples. LDS-PAGE of five gels was performed. Five strips of the identical length, but of varying width, were cut out of each gel and combined onto appropriate assembling filter papers (*AFP*, *#1* through *#5*)

6. The unit is completely assembled by adding the lid on the buffer core, and connected to a power supply. Transfer the proteins at 30 V constant for 90 min.

3.6 Immunoblotting and Chemiluminescent Detection

1. After transfer is stopped, remove the membrane out of the blot module and attach to the middle of a square Petri dish by stick-

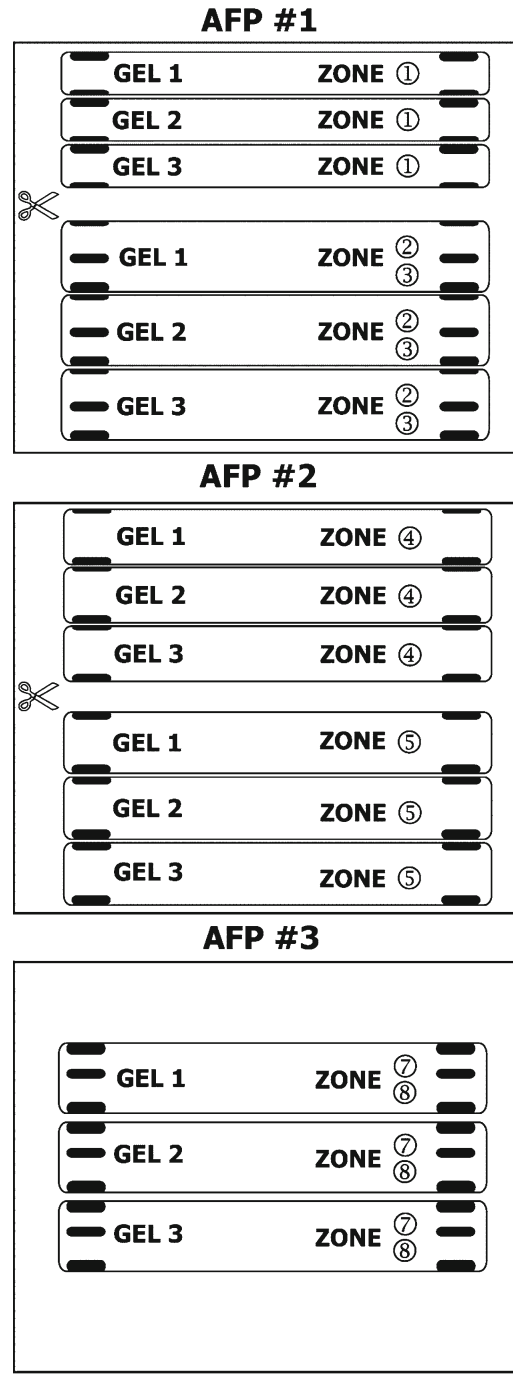

Fig. 5 A strategy for assembly of five gel strips following LDS-PAGE of three sets of samples. LDS-PAGE of three gels was performed. Five strips of the identical length, but of varying width, were cut out of each gel and combined onto appropriate assembling filter papers (*AFP #1* through *#3*)

ing the membrane's corners with a transparent tape. Discard used filter papers and gel strips.

2. Equilibrate the membrane by rinsing with dH_2O for 3–5 min. Discard dH_2O and incubate the membrane with 25–30 mL of Blocking buffer for 1 h at RT on a rotating platform.
3. After the blocking step, briefly rinse the membrane with dH_2O and incubate with appropriate 1°Ab solution at dilution ratio as recommended by a manufacturer overnight at +4 °C on a rotating platform (*see* **Note 15**).
4. Extensively rinse the membrane with dH_2O and wash four times for 7 min each with 1× TBS-T buffer at RT on a rotating platform.
5. Incubate the membrane with appropriate 2°Ab at dilution ratio as recommended by a manufacturer for 1 h at RT on a rotating platform followed by **step 10** once again. We routinely use Horse Anti-Mouse HRP-linked IgG 2°Ab (Cell Signaling, Danvers, MA, #7076) at 0.1 μL/mL concentrations and Goat Anti-Rabbit HRP-conjugated IgG (H + L) 2°Ab (Thermo Scientific, #31460) at 0.025 μL/mL concentration.
6. Incubate the membrane with a working solution of ECL reagent for 5 min. Place the membrane upside down in the precut piece of Sheet protector and place in the Imaging system.
7. Capture and quantify the signal intensity of protein bands using KODAK Digital Science software (*see* **Note 16**). If the researcher wants to compare the signals from different blots, then the capture time and number of frames should be equal for each separately exposed membrane.

3.7 Application Examples

Challenge 1. To compare the activation kinetics (e.g., at 0, 1.5, 3, 5, 7.5, 10, 20, 30 min) of protein of interest (e.g., ERK1/2) in control (K) and under perturbed conditions (e.g., in the presence of the Phosphoinositide 3-kinase inhibitor wortmannin, WT) in cells that received different strengths of stimulation (e.g., 0.02, 0.2, 2, and 20 nM EGF).

Solution. For electrophoresis, the samples may be loaded using four different strategies (Fig. 6a, b). After separation of proteins, each out of eight gels can be cut into desired number of strips. The given experimental task requires analyzing the activating phosphorylation of ERK1/2 (Zone ⑤, p-ERK1/2 (T202/Y204), 42/44 kDa) by the upstream kinase. In addition, it may be desired to check the activity of ERK1/2 by measuring the phosphorylation status of its immediate downstream target p90 ribosomal S6 kinase (Zone ③, p-p90RSK (S380), 90 kDa). Since the cells were

treated with phosphoinositide 3-kinase (PI3K) enzyme inhibitor WT, it is important to verify the efficiency of PI3K inhibition. There are many pleckstrin homology (PH) domain-containing proteins whose recruitment to the plasma membrane and subsequent activation/phosphorylation may be altered due to depletion of PIP_3, which is generated only by active PI3K. One of such proteins is a serine/threonine kinase Akt (Zone ④—p-Akt (S473), 60 kDa). S6 ribosomal protein (Zone ⑦⑧, p-S6RP (S240/244), 32 kDa) is a common substrate for p70 S6 kinase (Zone ③④,

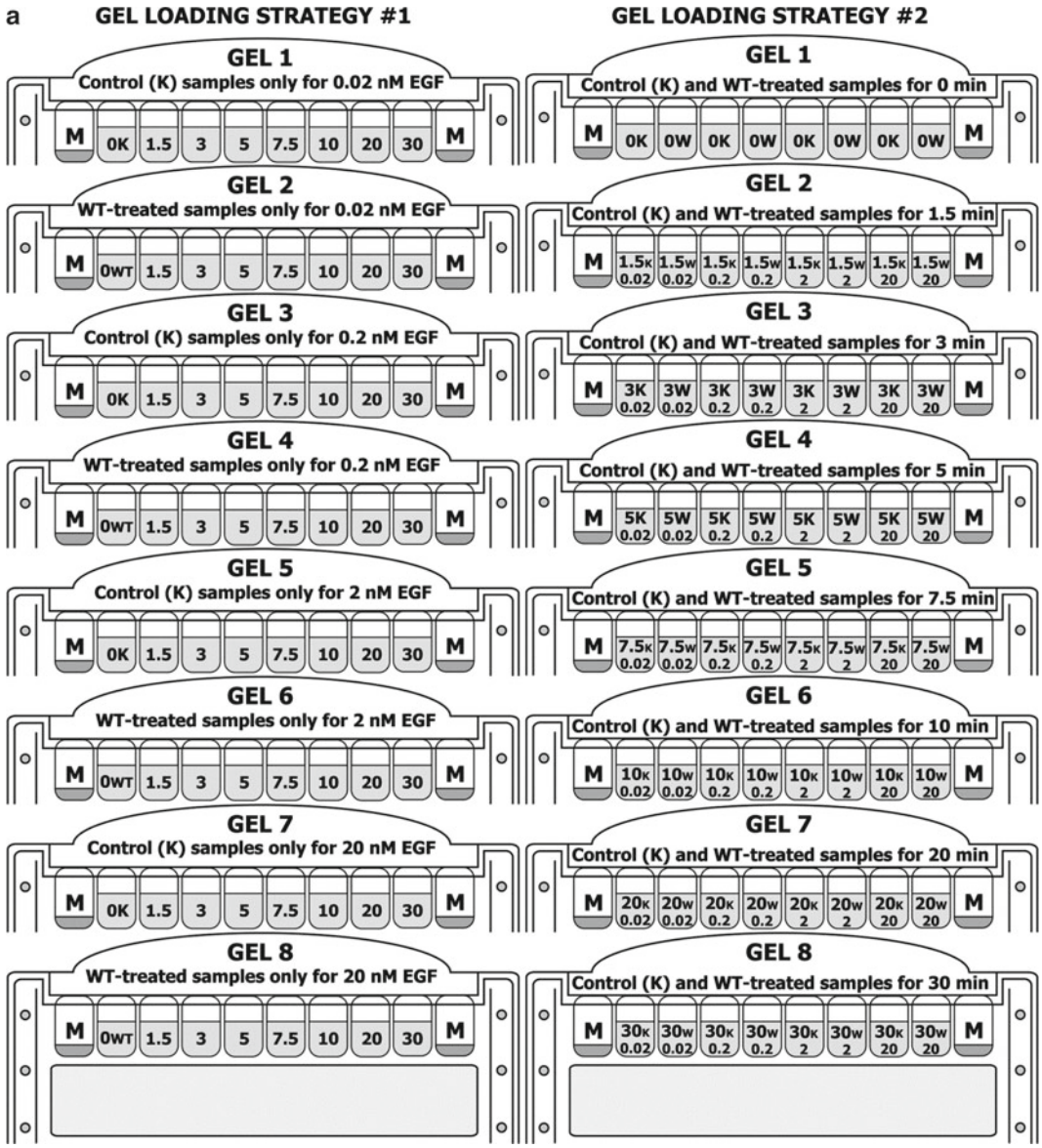

Fig. 6 Various Gel loading strategies (A—#1 and #2; B—#3 and #4) for electrophoresis of eight sets of samples

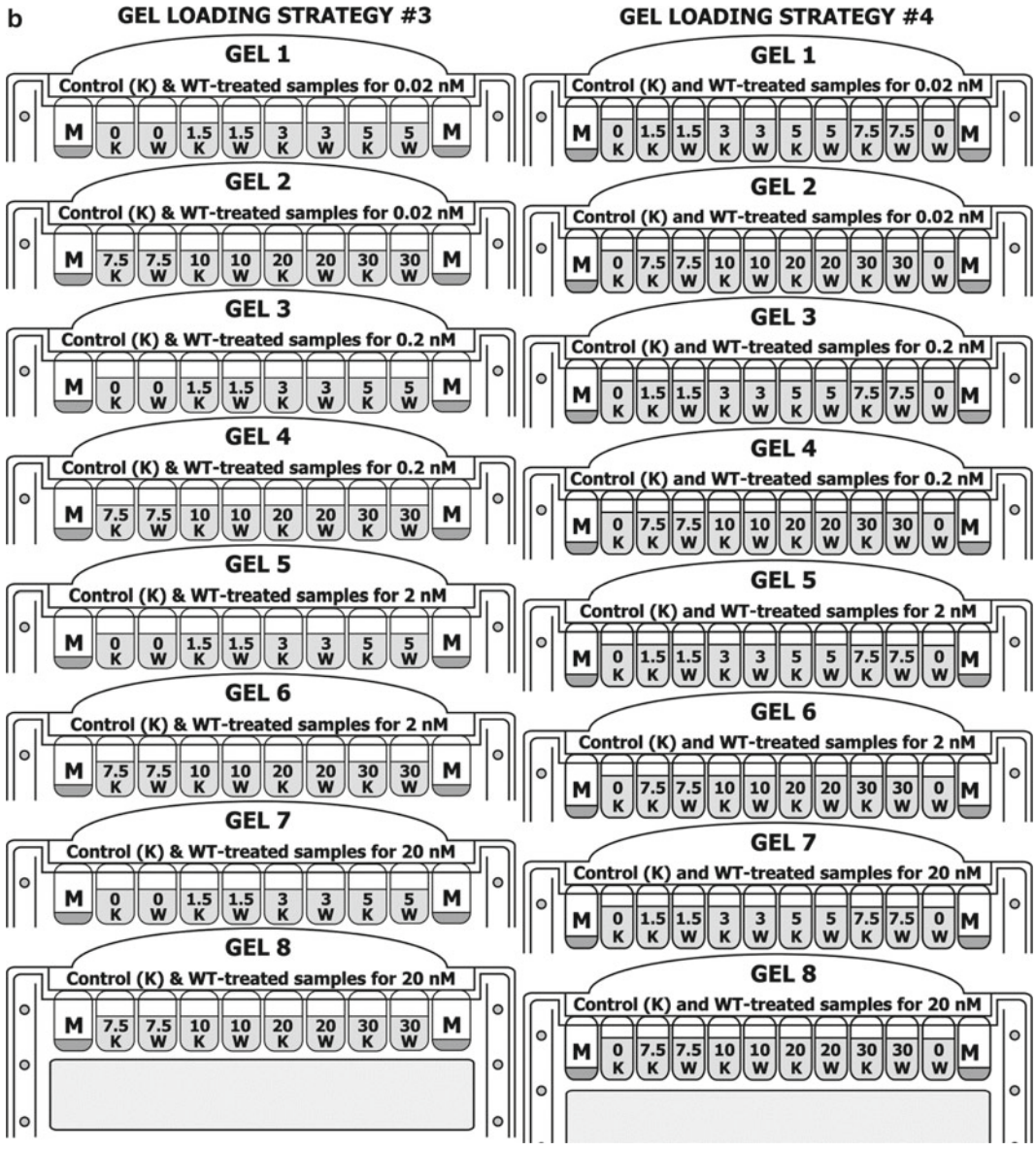

Fig. 6 (continued)

70–85 kDa) and p90RSK, and thus may serve as integrated readout of PI3K and ERK pathway activity. Moreover, it may be beneficial to check whether the inhibitor affects the EGF receptor (Zone ①—p-EGFR (Y1173), 160 kDa), which lies upstream of all signaling pathways that respond to EGF stimuli. Ideally, one may need to have a protein loading control. Unfortunately, actin (45 kDa), tubulin (50–55 kDa), and GAPDH (37 kDa) migrate in the Zones that, if cut out of the gel, would prevent further

detection of p-Akt and p-ERK. However, there are lower-molecular weight proteins that are expressed at steady-state levels (at least within a 30 min time frame of experiment) and thus may serve as good protein loading control not only for total cell lysates (e.g., Grb2 (Zone ⑥⑦, 25 kDa)) but also for separated cellular fractions: mitochondrial (COX IV, Zone ⑧⑨, 17 kDa; VDAC, Zone ⑥, 32 kDa), nuclear (Histone H3, Zone ⑧⑨, 17 kDa), plasma membrane (Ras, Zone ⑦⑧, 21 kDa), or caveolin-rich plasma membrane domains (caveolin-1/2, Zone ⑥⑦⑧, 21–24 kDa) (*see* Table 13). Gel cutting into six strips for analysis of p-EGFR, p-p90RSK, p-Akt, p-ERK, p-S6RP, and COX IV is illustrated in the *left upper panel of* Fig. 7.

Please note that if the strip for Zone ⑤ is cut out along with the strips from the upper and lower neighboring Zones (i.e., Zone ④ and Zone ⑥), then the gel cutting knife should pass through the bottom or the center of 50 and 37 kDa MW marker band (Fig. 7, *left middle panel*, *A*), depending how far away from the MW band migrates the next protein of interest. If the strip for Zone ⑤ is cut out only with the upper neighboring Zone ④, then the cutting may be done slightly below 37 kDa MW marker band (Fig. 7, *left middle panel*, *B*). If the strip for Zone ⑤ is cut out solely, then the cutting should encompass whole bands of MW marker to ensure risk-free detection of your protein of interest (Fig. 7, *left middle panel*, *C*). However, in this case the strip width will significantly increase, which would require aligning the strips onto a larger (e.g., extra-thick wide) AFP (Fig. 7, *right upper panel*).

Left lower panel of Fig. 7 shows the actual blot of p-ERK1/2 following the MSWB procedure performed with the protein samples that were loaded onto nine 10-well gels using Gel loading strategy #2 (Fig. 6), while the example of a blot derived from a more traditional Gel loading strategy #1 using four 10-well gels is shown in the *right middle panel of* Fig. 7. All four Gel loading strategies are suitable for densitometric analysis of the intensities of signals *upon equal conditions* including the incubation time of blot in ECL substrate, exposure time, and distance of the blot from the CCD camera. Obtained signals further can be normalized for protein loading and plotted onto a scatter chart for a comparative analysis of two or more groups of biological samples (Fig. 7, *right lower panel*, *AU—arbitrary units*). The signals may be expressed as fold over basal level, a percentage of the maximal signal intensity values for the respective phosphorylated proteins, a percentage of phosphorylated protein signal intensity to total protein, or as arbitrary units.

Gel loading strategy #4 (Fig. 6b) may be more convenient in case when protein activation is expressed as fold over the basal level, since each strip contains a separated control and inhibitor-

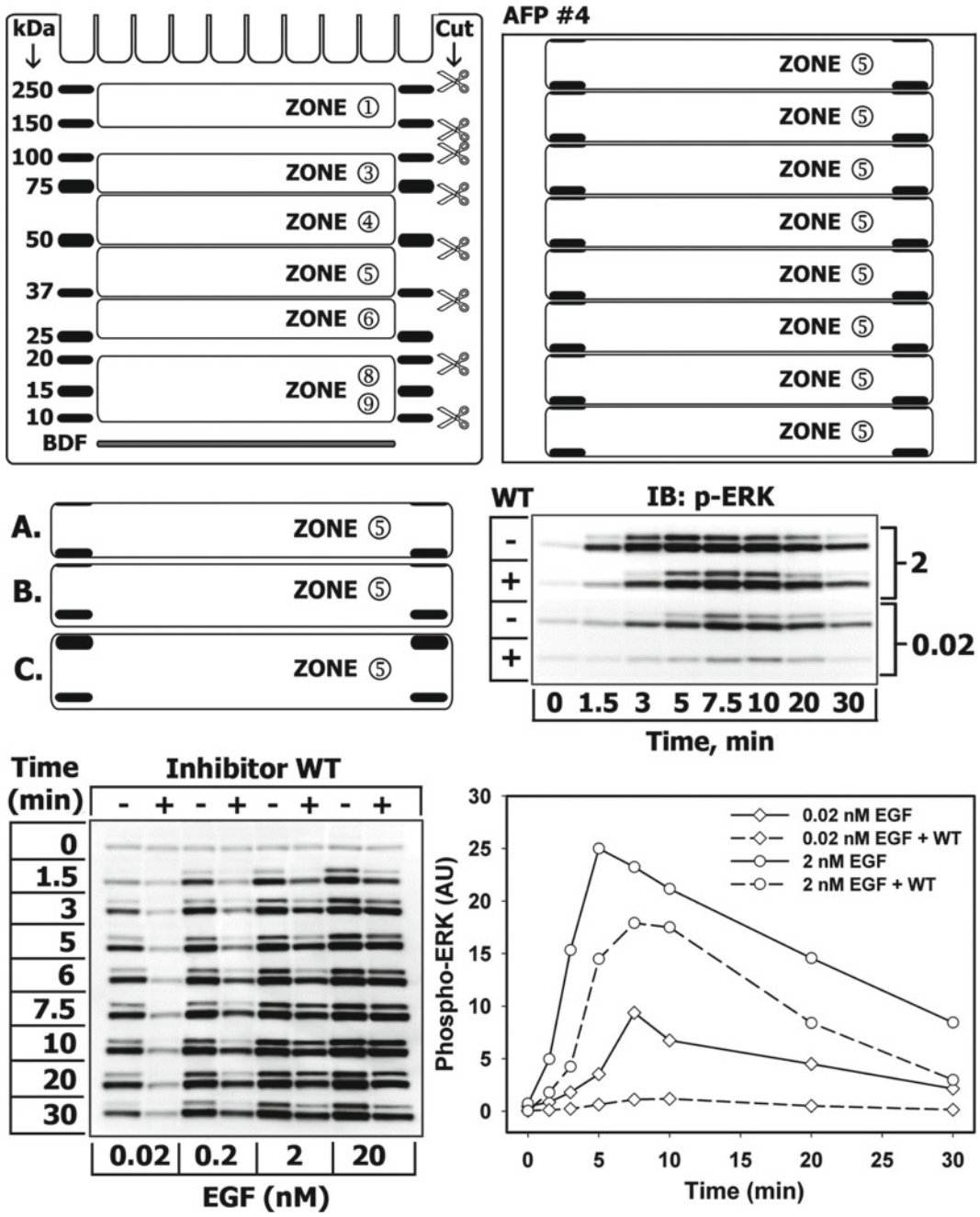

Fig. 7 MSWB strategy for concurrent measurement of EGF-induced ERK1/2 activation kinetics in response to inhibition of PI3/Akt cell survival signaling pathway

treated sample of nonstimulated cells. 0 K and 0 W may be the same sample loaded over and over again throughout all eight gels; biological duplicates (e.g., 0 K_1, 0 K_2 and 0 W_1, 0 W_2) loaded as technical quadruplicates; or biological quadruplicates

loaded in technical duplicates (0 K_1, 0 K_2, 0 K_3, 0 K_4 and 0 W_1, 0 W_2, 0 W_3, 0 W_4). Upon Gel loading strategies #1–3 (Fig. 6a, b), 0 K and 0 W can represent the same sample loaded in technical quadruplicate, a biological duplicate loaded in technical duplicate or biological quadruplicate. Their signals later should be averaged, respectively. A repetitive sample loading in 12-well gel (*see 7.5 min in all gels of* Fig. 7, *right panel*) can be avoided by including an extra time-point during the experimental procedure, (e.g., 6 min). However, for visual representation of the blot showing full continuous kinetics, the repetitive region with bands should be merged using Photoshop software. No merging of blot image is needed if the samples are visualized following Gel loading strategies #1 and #2 (Fig. 6a).

Challenge 2. To compare the activation kinetics (e.g., at 0, 5, 15, and 30 min) of EGFR, FAK, Akt, ERK1/2, and Histone 3 (H3) in untreated cells (control, *K*) and in cells that were pretreated with six different agents (*A*–*F*) prior to stimulation with 5 nM EGF.

Solution. For electrophoresis, the samples were loaded onto six 10-well Novex 4–12 % Bis-Tris gradient gels as shown in left upper panel of Fig. 8. Each out of six gels with separated proteins was cut into six strips containing indicated Zones for proteins of interest as shown in the *right upper panel*. Gel strips were then transferred onto appropriate AFP sheets and horizontally aligned together with identical protein Zone strips derived from the other five gels (*right middle panel*). After protein transfer and blocking steps, the membrane bearing protein Zone ① was probed with 1°Ab against p-EGFR (Y1173) (160 kDa; Cell Signaling #4407 at 1:1,000), membrane with Zone ②③—p-FAK (Y397) (125 kDa; Cell Signaling #8556 at 1:1,000), Zone ④—p-Akt (S473) (60 kDa; Cell Signaling #4051 at 1:1,000), Zone ⑤—p-MEK (S217/221) (Cell Signaling #9154 at 1:1,000), Zone ⑥⑦—Grb2 (25 kDa; Santa Cruz Biotechnology #sc-255 at 1: 500), and Zone ⑧⑨—p-H3 (S10) (17 kDa; Cell Signaling #9701). The resulting blots of Zone ④ (*left lower panel*) and Zone ⑥⑦ (*right lower panel*) after respective protein detection by chemiluminescence are shown to demonstrate the increased throughput, expected final signal/noise ratio, and sample-to-sample reproducibility for comparative study of cell signaling events under perturbed and unperturbed conditions. Please note that half of the first and the last lanes containing resolved MW marker are obscured from the visual field (*left and right lower panels*) by zooming in the CCD camera, because some antibodies tend to cross-react with marker proteins.

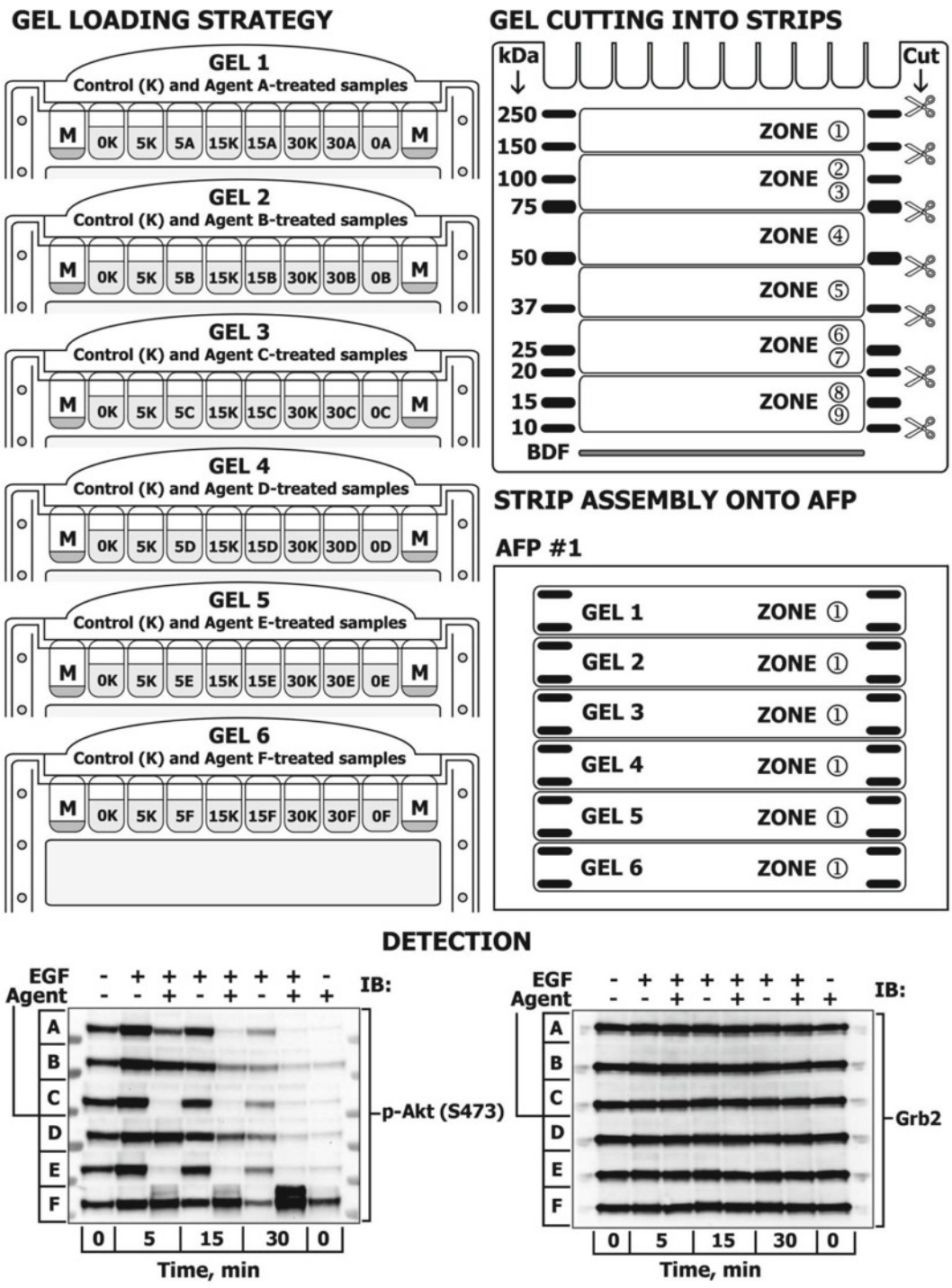

Fig. 8 Major MSWB steps for simultaneous analysis of temporal expression of different proteins engaged in EGFR signaling network

4 Notes

1. Pre-made lysis buffers, containing different detergents and additives that vary in their ability to facilitate cell/tissue solubilization as well as to extract and denature proteins or preserve protein–protein interactions, are commercially available.
2. Laemmli instead of LDS sample buffer can be used with appropriate running and transfer buffers. Electrophoresis can be performed under reducing (add DTT or β-mercaptoethanol) as well as nonreducing (omit adding reducing agent) conditions. Please note that under nonreducing conditions proteins may migrate differently than your MW marker.
3. Choose the type of apparatus for electrophoresis and protein transfer suitable for the size of your gels (e.g., mini-, midi-, or maxi-gels). If iBlot® Dry Blotting System is used, the transfer time can be reduced from 1.30 h to ≤7 min, but it requires further optimization of actual performance by user.
4. This protocol can be adapted for gels of any percentage, composition, size, and number of wells. For instance, lower percentage Bis-Tris or Tris-Acetate gels may be selected for separation of large MW proteins of interest. We routinely use gradient Bis-Tris gels that, when compared to Laemmli system's Tris-Glycine SDS-PAGE gels, have longer shelf-life; their neutral pH environment minimizes protein modifications and significantly delays acrylamide hydrolysis resulting in high-resolution separation of small to mid-size MW protein bands that are sharper and rarely overlap.
5. The researcher may adapt this protocol for various protein MW markers, but it will require further optimization by user. The reference bands of protein MW marker other than that used in this protocol will have different migration patterns, generating a different number of designated protein migration Zones that may also vary in width.
6. The researcher may adapt this protocol for other NuPAGE® Running buffers (MES-SDS and Tris-Acetate-SDS). Be aware that it will change the width and/or quantity of designated protein migration Zones depicted in this procedure and will require further optimization by user.
7. If desired, PVDF or nylon membranes can be also used. Most proteins can be successfully blotted using a 0.45 μm pore size membrane; however, for proteins of low molecular weight or peptides, a 0.2 μm pore size membrane is recommended. We have successfully used 0.2 μm for proteins with MW ranging from 14 to 220 kDa without compromising the efficiency of their transfer onto the membrane.

8. For more flexibility in handling, higher working volumes and higher rotating speeds, we prefer using individual over multiwell square dishes.
9. The samples to be loaded can be different or repetitive.
10. The table is designed to track the statistics of certain protein MW marker migration patterns in the gel of selected percentage under certain buffered conditions. The statistics is required for successive Multistrip Western blotting procedures if one needs to cut out the gel strip(s) containing protein(s) of interest of known MW, but no prestained marker has been loaded onto a gel.
11. The maximal number of gel strips that can be combined onto a single AFP depends on the overall dimension of the transfer unit, hence on the size (length and width) of AFP. Routinely we use *extra-thick narrow*, *medium*, *or wide* filter sheets that provide space for maximum of 10–11 strips of 0.7 cm width each. However, regularly we place fewer amounts of gel strips (e.g., six), especially when they are wider and/or the membrane should be cut into two or more pieces after electrophoretic protein transfer.
12. If some pauses occur, regularly wet the surface of gel strips by dropping dH_2O.
13. Alternatively, the whole piece of nitrocellulose membrane can be treated with blocking reagent and then incubated with the mixture of primary antibodies (be sure that they do not cross-react) in a single dish.
14. The pads should rise at least 0.5 cm over the rim of the cathode core. If not, then insert one or more thin filter paper sheets on the top of a thick filter paper in the tank.
15. 1°Ab can be collected into the 50 mL tube and reused several times if supplemented with 0.05–0.1 % (w/v) sodium azide. If precipitation occurs, filter the solution through 0.22 μm filter.
16. The chemiluminescent signal can be visualized by another imaging instrument and quantified using appropriate software. Alternatively, the signal can be captured on the film followed by densitometric quantification.

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