Multiplex protein detection in 2-D gel electrophoresis using the Amersham ECL Plex fluorescent Western blotting system

Key words: *ECL Plex • fluorescent Western blotting • multiplex detection • two-dimensional gel electrophoresis • post-translational modification*

We examined the performance of the ECL Plex™ Western blotting system in both 1-D and 2-D Western blotting applications. The 1-D Western blotting experiment showed that both the nonphosphorylated and phosphorylated forms of GSK3β could be detected in PC-3U cells without TGF-β activation. The 2-D Western blotting experiment gave information on phosphorylation states that could not be obtained by 1-D Western blotting. A 48-kDa band corresponding to phosphorylated GSK3β in the 1-D experiments was resolved into at least five distinct protein isoforms, two of which were phosphorylated at serine 9.

Introduction

The Amersham™ ECL Plex Western blotting system based on sensitive CyDye™ conjugated secondary antibodies is most commonly used after separation of proteins on one-dimensional (1-D) SDS-PAGE gels. ECL Plex Western blotting provides sensitivity, linearity, and a dynamic range of nearly four orders of magnitude (1), but also the possibility of multiplex analysis—detection of two protein epitopes on the same membrane simultaneously. Multiplexing is commonly used for the quantitation of one protein relative to a protein of known abundance (housekeeping protein), but can also be used for the detection and quantitation of post-translational modifications such as phosphorylation, provided that there are antibodies available.

In this study, a two-dimensional (2-D) Western blotting approach was evaluated in addition to the traditional 1-D workflow, using fluorescent secondary antibodies, namely the ECL Plex Western Blotting Detection System. Human PC-3U cell lysate was separated by 1-D and 2-D SDS-PAGE, followed by blotting and detection of the beta form of glycogen synthase kinase-3 (GSK3β). ECL Plex CyDye-conjugated secondary antibodies were used for simultaneous detection of the nonphosphorylated and phosphorylated forms of GSK3β. The results show that the ECL Plex Western blotting system enables multiplexing of GSK3β and Ser 9–phosphorylated GSK3β on 1-D gels, and that the 2-D Western approach can give higher resolution information on different phosphorylation states.

Materials

Products used

ECL Plex goat-α-mouse IgG-Cy3, 150 µg	PA43009
ECL Plex goat-α-rabbit IgG-Cy5, 150 µg	PA45011
CyDye DIGE Fluor, Cy™2 minimal dye	RPK0272
Hybond-LFP™, 20 x 20 cm, 10 sheets	RPN2020LFP
ECL Plex Fluorescent Rainbow™ Markers, full range, 500 µl	RPN851
ECL Advance™ Blocking Reagent	RPN418
2-D Clean-Up Kit	80-6484-51
2-D Quant Kit	80-6483-56
Immobiline™ DryStrip pH 7-11 NL, 7 cm	17-6003-68
DeStreak™ Rehydration Solution	17-6003-19
IPG Buffer pH 7-11 NL	17-6004-39
IPG Buffer pH 3-10 NL	17-6000-88
Ettan™ IPGphor™ II IEF System	80-6505-03
miniVE Vertical Electrophoresis System	80-6418-77
EPS 301 Power Supply	18-1130-01
TE 22 Mini Tank Transfer Unit	80-6204-26
Typhoon™ 9410 and ImageQuant™ TL	63-0055-80
PlusOne™ Bromophenol Blue	17-1329-01
PlusOne DTT	17-1318-01
PlusOne Glycerol	17-1325-01
PlusOne Glycine	17-1323-01
PlusOne SDS	17-1313-01
PlusOne Tris	17-1321-01

Other materials required

GSK3 beta antibody [G8] (abcam)	ab2602
Phospho-GSK-3beta (Ser9) Antibody (Cell Signaling)	9336S
10x PBS (Medicago)	12-9423-5
Novex™ 12% Tris-glycine gel (Invitrogen)	EC60055BOX
Novex 12% Tris-glycine gel, 2D well	EC6006BOX
Methanol (Merck)	K33730207
Tween™ 20 (Merck)	8.22184.1000
99.8% anhydrous dimethylformamide (Sigma-Aldrich)	705-6
Lysine (Sigma-Aldrich)	L-5626

Human prostate carcinoma (PC-3U) cells, a subclone of PC-3 [human prostate cancer cells (nr 3); ATCC number CRL-1435] modified for enhanced TGF-β receptor production (2)

Methods

1-D gel electrophoresis

Human PC-3U cells were lysed in a RIPA buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 10% glycerol, 1% NP40, 0.5% deoxycholate) and loaded onto Novex 12% Tris-glycine gels in a series of four-fold dilutions from 30 µg to 0.47 µg of total protein. Gel electrophoresis was performed for 2.5 h at 100 V using the miniVE Vertical Electrophoresis System.

2-D gel electrophoresis

PC-3U cell lysate was further prepared for 2-D electrophoresis by using the 2-D Clean-Up Kit for desalting and cleanup. Protein concentration was measured with the 2-D Quant Kit. PC-3U cell lysate corresponding to 50 µg total protein was labeled with 400 pmol of CyDye DIGE Fluor, Cy2 minimal dye for 30 min on ice. The reaction was stopped by the addition of 10 mM lysine.

For each Immobiline DryStrip gel, unlabeled PC-3U cell lysate (25 µg of protein) was mixed with Cy2-labeled PC-3U cell lysate (5 µg of protein).

First-dimension electrophoresis was performed on 7-cm Immobiline DryStrip pH 7-11 gels, selected because the predicted pI of GSK3β is 8.95. A mixture of labeled and unlabeled human PC-3U cell lysate (30 µg of protein) was applied to the rehydrated strips by cup loading, and the samples were focused for 11 kVh on the Ettan IPGphor II instrument (3).

Second-dimension SDS-PAGE was performed in the MiniVE Vertical Electrophoresis System using Novex 12% Tris-glycine gels with 2-D wells. ECL Plex Fluorescent Rainbow Markers were loaded onto the gel for size indication. Protein samples were first allowed to enter the gel gently for 15 min at 15 mA per gel, followed by separation for 2 h at 30 mA per gel.

Protein blotting and fluorescent detection

After electrophoretic separation the gels were transferred to Hybond-LFP membranes for 2.5 h at 25 V using a TE 22 Mini Tank Transfer Unit, followed by incubation in 2% ECL Advance Blocking Reagent in TBS overnight at 4°C. The blots were then incubated with rabbit polyclonal anti–phospho-GSK3β (Ser9) and mouse monoclonal anti-GSK3β primary antibodies (1:1000 dilution in TBS + 0.1% Tween-20; TBST) overnight at 4ºC. The blots were washed twice quickly, then twice for 5 min each in TBST. They were then incubated for 1 h, protected from light, with the secondary antibodies: ECL Plex goat-α-rabbit IgG-Cy5 and ECL Plex goat-α-mouse IgG-Cy3 (1:2500 dilution in TBST). The membranes were washed three times quickly, then four times for 5 min each in TBST, followed by two brief washes in TBS. Membranes were dried at 40°C for 1 h before scanning.

Imaging was performed on the Typhoon 9410 scanner using the 633-nm (red) laser with a 670BP30 filter for Cy5-conjugated antibodies and the 532-nm (green) laser with a 580BP30 filter for Cy3 conjugates. The PMT value was adjusted until the most intense band nearly reached saturation. The images were then analyzed using ImageQuant TL software.

Application

Lysates of human PC-3U cells were prepared, run on 1-D and 2-D gels, transferred to Hybond-LFP membranes, and then probed with antibodies according to the protocol previously described. The targeted proteins were the non-phosphorylated and phosphorylated forms of GSK3β (GSK3β and pGSK3β, respectively). As primary antibodies we used mouse monoclonal anti-GSK3β targeted with ECL Plex goat-α-mouse IgG-Cy3 and rabbit polyclonal anti–phospho-GSK3β (Ser9) targeted with ECL Plex goat-α-rabbit IgG-Cy5.

Results and discussion

One-dimensional gel electrophoresis

Using a mixture of ECL Plex goat-α-mouse IgG-Cy3 and ECL Plex goat-α-rabbit IgG-Cy5 fluorescent antibodies, GSK3β and phospho-GSK3β (Ser9) were simultaneously detected in one blot (Fig 1). The anti-GSK3β primary antibody recognized two bands at about 48 and 51 kDa (Fig 1A). The 48-kDa band corresponds to GSK3β and the 51-kDa band corresponds to GSK3α, a result of cross-reactivity of the primary antibody, as stated by the manufacturer. The anti-pGSK3β primary antibody also recognized two protein bands at 48 and 49–50 kDa (Fig 1B). The 48-kDa band corresponds to GSK3β phosphorylated at serine 9. The 49- to 50-kDa band appeared somewhat fuzzy, not migrating as one distinct protein isoform. This may be because it corresponds to different phosphorylation states of GSK3β, migrating differently in the gel. Another explanation could be that the 49- to 50-kDa band is nonspecific, that it does not correspond to GSK3β.

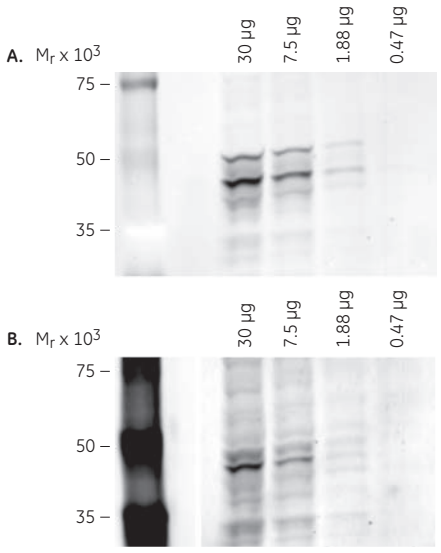

Fig 1. 1-D electrophoresis of PC3U cell extract. A: ECL Plex Cy3-conjugated secondary antibody targeted against GSK3β; B: ECL Plex Cy5-conjugated secondary antibody targeted against the phosphorylated form of GSK3β (pGSK3β).

Figure 2 displays the same images as seen in Figure 1, but in color: images resulting from scanning with the green laser for GSK3β (Fig 2A), the red laser for pGSK3β (Fig 2B), and the overlay of scans from GSK3β (Cy3, green) and pGSK3β (Cy5, red) (Fig 2C). The yellow color of the 48-kDa band shows that the signals from the two primary antibodies overlap: the 48-kDa band contains phosphorylated GSK3β.

TGF-β is a potent growth factor for stimulating a number of cellular responses, one of them resulting in the phosphorylation of GSK3β (4). TGF-β activation will induce phosphorylation of GSK3β significantly, but for this study only unactivated PC-3U cell lysate was used. We could detect phosphorylation without any TGF-β activation; this can be explained by the ongoing dynamic process of phosphorylation/dephosphorylation in the cell. The overlay image of anti-GSK3β and anti-phospho-GSK3β (Ser9) scans clearly shows that a population of GSK3β is phosphorylated in PC-3U cells without TGF-β activation.

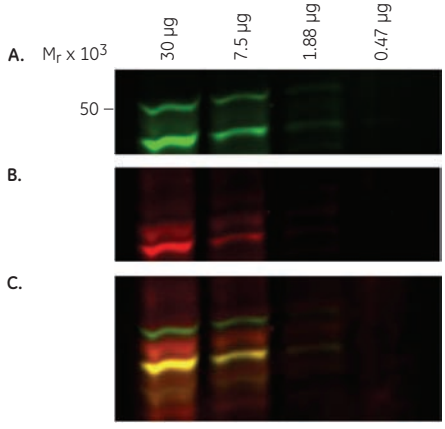

Fig 2. Color images of 1-D Western blot scans from Fig 1. A: ECL Plex Cy3-conjugated secondary antibody targeted against GSK3β; B: ECL Plex Cy5-conjugated secondary antibody targeted against the phosphorylated form of GSK3β; C: overlay of A and B.

Two-dimensional gel electrophoresis

Two dimensional gel electrophoresis in mini-format allows high resolution, using both the pI and the molecular weight of proteins for separation, while the small format makes it convenient and fast compared to large-format 2-D electrophoresis. In this application, a fraction of the PC-3U cell lysate used for 2-D electrophoresis was prelabeled with CyDye DIGE Fluor Cy2 minimal dye to allow for direct visualization of total protein without post-staining of the membrane. Figure 3A shows the Cy2, Cy3, and Cy5 scans overlaid—total protein (blue), GSK3β (green) and pGSK3β (red). Figure 3B shows only the two forms of the target protein, GSK3β (green) and pGSK3β (red). Different epitopes at the same position detected by both antibodies appear yellow (Figs 3A and 3B). The positions of the detected spots agree with the predicted pIs of 8.95 and 8.98 and with the molecular weights of 48 and 51 kDa for the α and β forms of GSK3, respectively (http://au.expasy.org/tools/pi_tool.html).

Different phosphorylation states on 2-D gels are displayed as spot trains depending on the number of different states, where one phosphate will cause a shift towards the anode and a small increase in size (80 Da). GSK3β has six potential phosphorylation sites (Ser9, Ser21, Thr43, Tyr216, Ser389, and Thr390; Cell Signaling). Using an isoelectric point calculator (5), the predicted pIs of GSK3β nonphosphorylated and phosphorylated at one to six phosphorylation sites are 8.98 for nonphosphorylated GSK3β, then 8.90, 8.81, 8.71, 8.59, 8.44, and finally 8.25 for the putative fully phosphorylated GSK3β. It is likely that the spot trains seen in Figures 3A–C correspond to different phosphorylation states of GSK3 isoforms. The molecular weight of the two protein spots corresponding to phosphorylated GSK3β in Figure 3 does not correspond to the molecular weight of the 49- to 50-kDa band seen in Figures 1B, 2B, and 2C, indicating that the 49- to 50-kDa band in the 1-D approach is not related to GSK3β.

The results from the 2-D Western approach show that the 48-kDa band corresponding to phosphorylated GSK3β in the 1-D experiments could be resolved into at least five distinct protein isoforms in the 2-D experiment, where two isoforms were phosphorylated at serine 9 (recognized by the anti-phospho-GSK3β (Ser9) primary antibody).

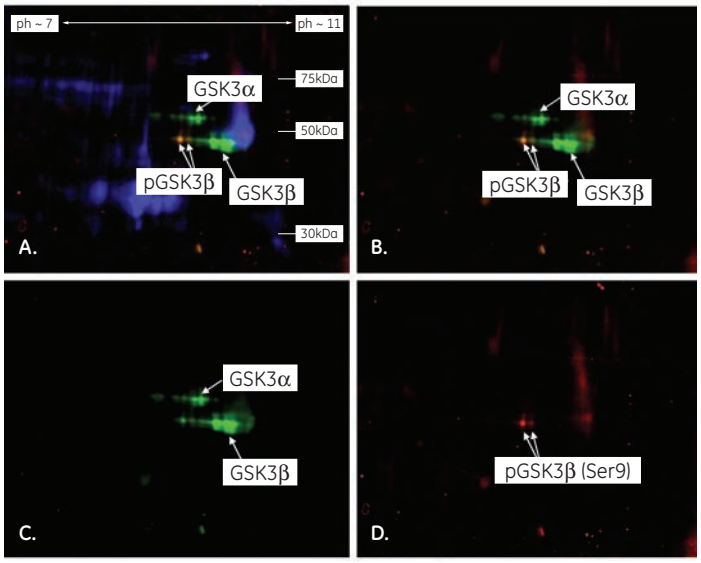

Fig 3. Images of 2-D Western blot scans. A: Overlay of Cy2, Cy3, and Cy5 images; B: Overlay of Cy3 and Cy5 images; C: Cy3 image; D: Cy5 image. Total protein (Cy2, blue), ECL Plex Cy3-conjugated secondary antibody targeted against GSK3β (green), and ECL Plex Cy5-conjugated secondary antibody targeted against the phosphorylated form, pGSK3β (red). Molecular weights and pH range are shown in panel A. Protein spots representing GSK3β (48 kDa), GSK3α (51 kDa), and the serine 9–phosphorylated form of GSKβ are indicated.

Conclusions

The high level of performance of the ECL Plex Western blotting system in both 1-D and 2-D Western blotting applications has clearly been demonstrated in this study. The 1-D Western blotting experiment showed that both the nonphosphorylated and phosphorylated forms of GSK3β could be detected in PC-3U cells without TGF-β activation.

The 2-D Western blotting experiment gave additional information on phosphorylation states compared to 1-D Western blotting. The 48-kDa band corresponding to phosphorylated GSK3β in the 1-D experiments could be resolved into at least five distinct protein isoforms, two of which were phosphorylated at serine 9.

Acknowledgment

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References

1. Application note: Multiplex protein detection using the ECL Plex fluorescent Western blotting system, GE Healthcare, 28-4015-40, Edition AA (2005).
2. Franzén, P. *et al.* Different signals mediate transforming growth factor-beta1-induced growth inhibition and extracellular matrix production in prostate carcinoma cells. *Exp. Cell Res.* **207**, 1–7 (1993).
3. 2-D Electrophoresis, Principles and Methods, GE Healthcare, 80-6429-60, Edition AC (2005).
4. Edlund, S. *et al.* Interaction between Smad7 and beta-catenin: importance for transforming growth factor beta-induced apoptosis. *Mol. Cell. Biol.* **25**, 1475–1488 (2005).
5. Bjellqvist, B. *et al.* The focusing positions of polypeptides in immobilized pH gradients can be predicted from their amino acid sequences. *Electrophoresis* **14**, 1023–1031 (1993).

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