Journal of Chromatography A, 1217 (2010) 7177–7181

Contents lists available at ScienceDirect

Journal of Chromatography A

journal homepage: www.elsevier.com/locate/chroma

Short communication

Repeated probing of Southwestern blots using alkaline phosphatase stripping

Yinshan Jia, Daifeng Jiang, Harry W. Jarrett*

Department of Chemistry, University of Texas San Antonio, San Antonio, TX 78249, USA

ARTICLE INFO

Article history:
Received 22 July 2010
Received in revised form 7 September 2010
Accepted 10 September 2010
Available online 17 September 2010

Keywords:
Southwestern blot
Alkaline phosphatase
Stripping
DNA-binding proteins
Transcription factor

ABSTRACT

Southwestern blotting is when a DNA sequence is used to probe DNA-binding proteins on an electrophoretic gel blot. It would be highly desirable to be able to probe a blot repeatedly with different DNA sequences. Alkaline phosphatase can remove 5′-phosphoryl groups from DNA and radiolabeled 5′-^{32}P-DNA probes are commonly used in Southwestern blotting. Here is shown that once probed, the radioisotope signal on the blot can be effectively removed by brief digestion with alkaline phosphatase, and the blot can then be repeatedly probed at least six times with different DNA probes. This exceeds the repetitions possible with another commonly used method using SDS. The technique can be used with either one-dimensional or multi-dimensional Southwestern blots and does not have a large effect on the phosphorylation state of the blotted proteins. An alternative method using T4 polynucleotide kinase stripping is also introduced but was less well characterized.

1. Introduction

Southwestern blotting is a method for detecting DNA-binding proteins. It is akin to other blotting methods in that proteins are separated by gel electrophoresis and the gel blotted to nitrocellulose, polyvinylidinedifluoride (PVDF) or other suitable media. In the case of Southwestern blotting, the proteins on the blot are next renatured, at least partially, and then a labeled duplex DNA is added and allowed to bind to proteins specific for that DNA sequence.

Southwestern blotting of proteins separated by one-dimensional SDS-PAGE has been commonly used to characterize transcription factors, proteins which bind DNA with high affinity (typically < nM) and regulate the transcription of genes. More recently, two-dimensional gel electropheresis (2DGE) Southwestern blotting has been reported and interfaced to LC–MS/MS to characterize transcription factors [1]. Multi-dimensional electrophoresis requires considerable additional effort and it would be highly desirable if the blots, once prepared, could be used multiple times for probing with additional DNA sequences. To do so, it is necessary to remove the labeled DNA probe, prior to probing with an additional sequence. Such methods to remove labeling are commonly called “stripping” methods. Unfortunately, the way most blots have been stripped is detrimental to their continued use and so blots can be re-used only a few times. With 2DGE-Sothwestern blotting, we tried six different stripping procedures and found only one, SDS stripping, effectively removed the probe DNA. However, SDS-stripping also required that the blot proteins be again renatured and was also shown to remove protein from the blots [1].

Here, we report a different approach to stripping. Calf intestinal alkaline phosphatase hydrolyzes phosphate esters and has been used to remove 5′-phosphoryl groups from DNA, for example in molecular biology [2]. If the probe DNA was 5′ end labeled by the polynucleotide kinase reaction, the label will be effectively removed by alkaline phosphatase digestion. This method of stripping is effective and allows for repeated reprobing of Southwestern blots.

2. Methods

2.1. Materials

Poly(2′-deoxyinosinic-2′-deoxycytidylic acid) (poly dI:dC, catalogue number 81349) was from Sigma Chemical Co. (St. Louis, MO, USA). Alkaline phosphatase (ALP, catalogue number 10 713 023 001, 1 unit/μl) from calf intestine was from Roche Diagnostics GmbH (Mannheim, Germany). Rabbit polyclonal phosphor-Ser antibody (catalogue number AB1607) was from Millipore (Bellerica, MA, USA). Mouse monoclonal phosphor-Tyr antibody (catalogue number SC-7020) was from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Goat anti-rabbit-horseradish peroxidase (catalogue number 6160-05) and goat anti-mouse IgG-horseradish peroxidase (1030-05) second antibodies were from Southern Biotechnology (Birmingham, AL, USA).

* Corresponding author at: Department of Chemistry, University of Texas San Antonio, 1 UTSA Circle, San Antonio, TX 78249, USA. Tel.: +1 210 458 7053; fax: +1 210 458 7428.
E-mail address: harry.jarrett@utsa.edu (H.W. Jarrett).

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doi:10.1016/j.chroma.2010.09.033

2.2. DNA and oligonucleotides

The 281 bp c-jun core promoter was produced by polymerase chain reaction as previously described [3]. All oligonucleotides were synthesized by Integrated DNA Technologies (Coralville, IA, USA). Oligonucleotide probes used are MEF2 (5′-tggGCTATTTTTAGGgttg-3′, c-jun promoter position −60 to −51), Jun/Ku (5′-ccgtGAGCCTCcgcgggcccag-3′, −176 to −157) and SMAD3/SMAD4 (5′-ggCAGACAGACAgacacag-3′, +70 to +83) and are all duplexes of the sequences shown annealed to their complementary strand. The response elements are shown in upper case letters.

2.3. Oligonucleotide labeling

DNA or oligonucleotides were 5′ end labeled with T4 polynucleotide kinase and 20 μCi γ-^{32}P-ATP in 20 μl. Excess ATP was removed by spin chromatography on a 1 ml P6 (BioRad Laboratories, Hercules, CA, USA) column and adjusted to approximately 20,000 counts per minute per pmol DNA.

2.4. Preparation of nuclear extract

HEK293 nuclear extract was prepared [4]. Total protein concentration measured by the Bradford protein assay [5] was 4.6 mg/ml.

2.5. Gel electrophoresis and Southwestern blotting

For one-dimensional Southwestern blots, 10 μg of nuclear extract was applied to 12% SDS-PAGE [6] using 0.075 cm × 7 cm × 10 cm mini-gels. For 2DGE was performed using the method of O'Farrell [7] as previously described [1]. Electroblotting to PVDF (Sequi-blot 0.2 μm, BioRad Laboratories) was in 10% methanol in 25 mM Tris, 192 mM glycine, overnight at 50 V at 4 °C as described [1]. After electroblotting, the polyvinylidene fluoride (PVDF) membrane was blocked overnight at 4 °C in 25 ml/blot (2.8 ml/cm^2) SW buffer (10 mM HEPES, pH7.5, 10 mM $MgCl_2$, 50 mM NaCl, 0.1 mM EDTA, 1 mM DTT, and 0.1% Tween-20) containing 5% non-fat dried-milk to allow the blotted proteins to partially renature. The blot was probed in SW buffer containing 2.5% of BSA, 2.5 μg/ml poly dI:dC, and 2 nM of γ-^{32}P-ATP labeled oligonucleotide at 4 °C overnight. The blots were then washed four times, each in 25 ml of SW buffer, allowing 15 min incubation for each wash. The DNA–protein binding results were visualized by exposing X-ray film overnight.

2.6. Alkaline phosphatase stripping

Blots were typically stripped by incubation with 5 units of alkaline phosphatase in 2.0-ml of SW buffer, containing 2.5% of BSA at 37 °C for 30 min.

2.7. SDS stripping

Blots were stripped for some experiments as previously described [1] by incubation for 10 min in 2% SDS, 62.5 mM Tris–HCl, pH 6.8, 100 mM 2-mercaptoethanol at 50 °C. After stripping, using either method (Sections 2.6 and 2.7), the blot was washed three times using 25 ml of SW buffer each and allowing 5–10 min per wash. The blots were examined by autoradiography and then returned to SW buffer containing 5% non-fat dried milk for at least 5 h prior to being used for reprobing.

2.8. Western blotting

The same amount of nuclear extract was applied to eight wells of a prepared 10% SDS-PAGE [6], blotted to nitrocellulose, and the blot cut into eight strips. The blot was then blocked in TTBS (20 mM Tris, pH 8, 0.1 M NaCl, 0.1% Tween-20) containing 5% non-fat dried milk. The blots were incubated overnight at 4 °C in TTBS with the anti-phosphoserine or anti-phosphotyrosine primary antibodies at 1:500 dilution. After washing thrice in TTBS, the blots were incubated for 1 h with a 1:10,000 dilution of goat anti-rabbit-horseradish peroxidase or 1:5000 goat anti-mouse IgG-horseradish peroxidase second antibodies as appropriate and detected by enhanced chemiluminescence (Pierce Chemical Company, Rockford, IL, USA, catalogue number 34076).

3. Results and discussion

3.1. Alkaline phosphatase effectively strips the signal from Southwestern blots and the blots can be reprobed

To determine effective methods for stripping, we began with the simpler one-dimensional Southwestern blots using mini-gels. Previously [8], we had shown that Southwestern blotting under these conditions is specific for high affinity DNA-binding proteins and have shown the identity of one of these [1]. While such mini-gel blots are of lower resolution than others reported [8], they are sufficient for the purpose here. In Fig. 1A, the effect of alkaline phosphatase treatment is shown. Treatment with as little as 5 units of alkaline phosphatase effectively removes the radiolabel from the protein-bound DNA probe. Lower amounts of alkaline phosphatase or shorter treatment lead to incomplete signal removal (data not shown). This treatment should have only removed the 5′ radiolabel from the DNA, leaving unlabeled DNA still bound. To determine if blots once treated can be re-used, the experiment in Fig. 1B was performed, this time using freshly labeled oligonucleotide. Before stripping (positive control), there is significant bound label. After stripping, the blots were either incubated with the same radiolabeled probe (Jun/Ku) or with the same counts per minute of γ-^{32}P-ATP used in oligonucleotide labeling. Clearly, reprobing with radiolabeled DNA (Jun/Ku) resulted in the same amount of DNA-binding as occurred before any stripping. Thus, alkaline phosphatase effectively removes the radiolabel from the probe DNA (Fig. 1A), and addition of fresh DNA probe can replace the now unlabeled DNA from the blotted protein to restore the signal. The addition of ATP shows that the signal is not arising by binding this labeling component but rather requires the radiolabeled DNA sequence. Additionally, the blots were not subjected to repeated renaturation for these experiments. Thus, treatment with alkaline phosphatase is not affecting the renaturation of the blotted proteins or their ability to bind DNA.

3.2. Alkaline phosphatase stripping can be used repetitively while SDS stripping is more limited

In Fig. 2, we show the results of repetitive stripping and reprobing with the same DNA sequence. For this experiment, all autoradiographs shown were exposed the same length of time. Each time after stripping, the absence of signal was confirmed (panel B). With alkaline phosphatase, the signal is consistently high (panel A) and with very little background even up to six stripping and reprobing cycles. In contrast, by the second cycle SDS stripping resulted in a significant degradation of signal and increased background.

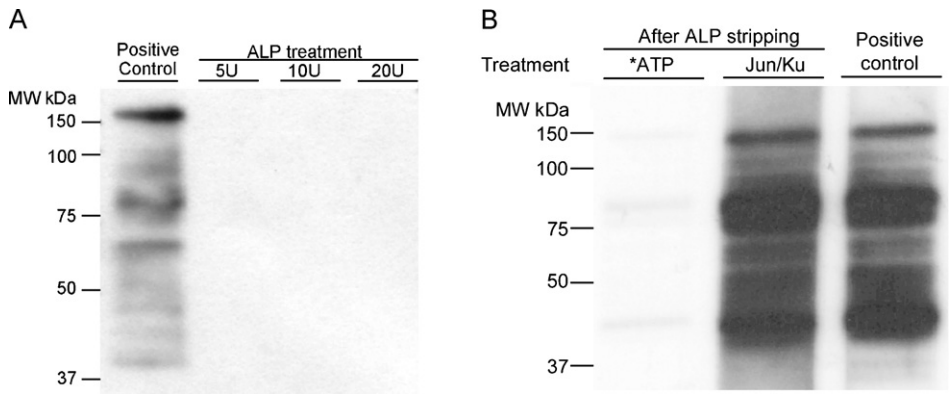

Fig. 1. Optimizing alkaline phosphatase dosage and reprobing analysis. (A) Alkaline phosphatase (ALP) stripping. The same amount (10 μg) of nuclear extract was applied to four wells of SDS-PAGE, blotted, and probed with the Jun/Ku oligonucleotide. The blot was then cut into individual wells. For the positive control, no stripping was performed and to the right is shown the result of stripping with 5, 10, and 20 units of ALP, respectively. (B) Similarly, the same amount of nuclear extract was applied to three wells of SDS-PAGE, probed with a freshly radiolabeled Jun/Ku oligonucleotide (Jun/Ku), and cut into individual wells. A well probed with Jun/Ku oligonucleotide but not stripped is shown to the right (positive control). For the other two well blots, after ALP stripping, the blots were either reprobed with the Jun/Ku oligonucleotide, or with an equal number of C.P.M. of γ-^{32}P-ATP (*ATP). In all case blots, if stripped, were stripped only once.

3.3. Reprobing using a different oligonucleotide probe

Figs. 1 and 2 both involved reprobing when the same DNA is used throughout for comparison purposes. However, a truly useful method would require that after probing with one DNA sequence, the blot can be reprobed with additional, different DNA probes and this is addressed in Fig. 3. Lanes 2 and 4 show the results when the blots were not stripped after probing with the MEF2 and Jun/Ku element oligonucleotides, respectively. These were chosen because there are clear differences in the bands labeled even though some are of similar migration behavior. After stripping in lanes 1 and 3, reprobing with a different oligonucleotide, MEF2 and Jun/Ku, respectively, the new oligonucleotide probe clearly gives the result closely matching that expected for that DNA sequence. Thus, the second, different probe gives the result expected for that probe and not the result that would be expected with the first probe. Put another way, after stripping, the results of the first probe are completely removed and a second probe can be used to investigate the binding of other sequences. Thus, probing with different DNA sequences is clearly feasible and accurate.

3.4. Alkaline phosphatase stripping has little effect on overall protein phosphorylation

Many transcription factors are also substrates for cellular protein kinases and this is important to regulating transcription [9–11]. Since alkaline phosphatase hydrolyzes phosphate esters, which includes protein phosphorylations, we next investigated the effect of stripping on the protein phosphorylation state of nuclear extract proteins. To investigate this, the endogenous phosphorylation of

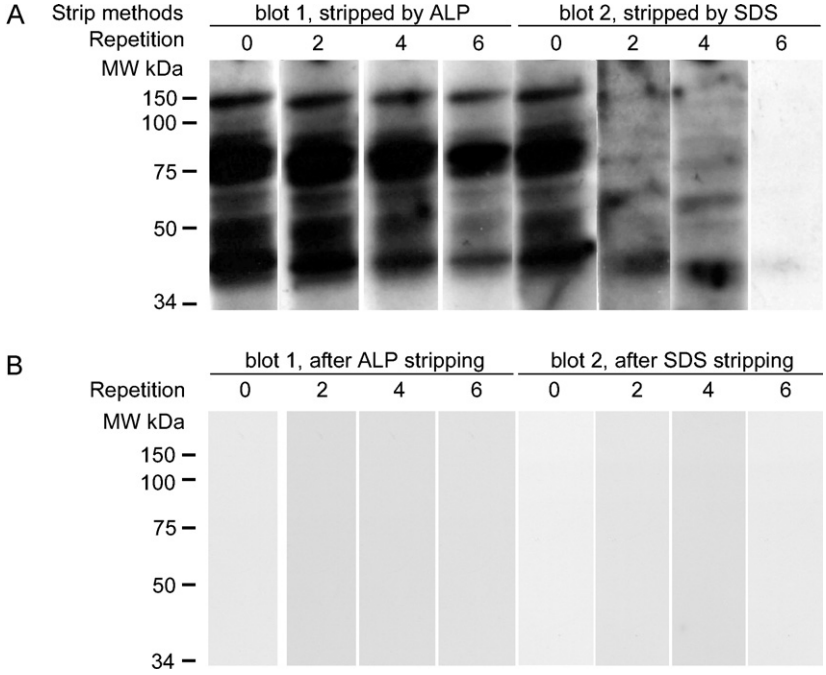

Fig. 2. Comparison of two stripping methods. Panel A, two different PVDF blots of SDS-PAGE fractionated HEK293 nuclear extract were used. Each blot is probed, stripped and reprobed up to six times. Using the alkaline phosphatase (ALP) stripping protocol the result is very similar even after the sixth stripping–reprobing cycle, while using the SDS stripping protocol, the signal degrades after a few cycles. Panel B, autoradiographs of the same blots as panel A after stripping showing that the signal is effectively removed at each cycle. All autoradiograms shown were exposed identically.

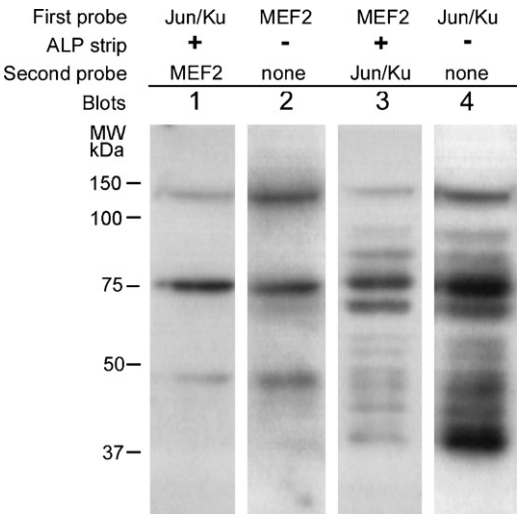

Fig. 3. Repeated probing with alternate oligonucleotides. Lanes 1 and 4 were probed with Jun/Ku oligonucleotide while lanes 2 and 3 were probed with MEF2 oligonucleotide. Lanes 1 and 3 were then stripped by ALP and reprobed with a different oligonucleotide, MEF2 oligonucleotide for lane 1 or Jun/Ku oligonucleotide for lane 3.

nuclear extract proteins was investigated using Western blotting with phosphoserine and phosphotyrosine antibodies (Fig. 4). When primary antibody is deleted (−) there is little staining while adding primary (+) antibody reveals multiple phosphorylated bands. Incubation in the buffer used for stripping alone (B) diminishes some of the background but otherwise has little effect and alkaline phosphatase stripping (ALP) in this buffer does diminish a few phosphorylated bands but overall has almost no effect. We conclude that while an individual protein phosphorylation may be affected, overall phosphorylation is not affected by the stripping procedure used.

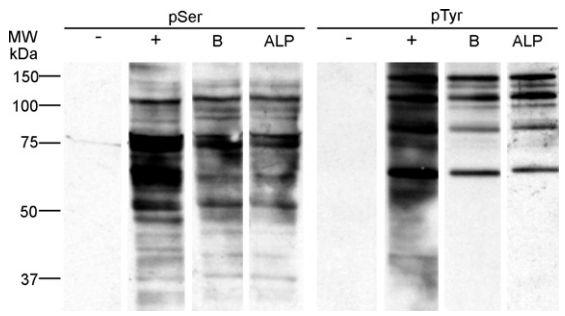

Fig. 4. ALP stripping does not greatly alter protein phosphorylation. The same amount of the same nuclear extract was applied to eight wells of SDS-PAGE, the gel blotted to nitrocellulose, and probed with Jun/Ku. The blot was then cut into strips for each well for western blotting. Four were used for the phosphor-Ser antibody and the other four for the phosphor-Tyr antibody. In each set, "−" denotes a western blot in which the primary antibody was left out, "+" and all other blots received both the primary and secondary antibodies. For "B", the blot was mock stripped by incubating in ALP buffer alone while "ALP" was incubated in buffer containing 5 units of ALP.

3.5. Alkaline phosphatase stripping is also applicable to 2DGE Southwestern blots

In Fig. 5, we performed a 2DGE Southwestern blot of nuclear extract, first probing with the entire 281 bp c-jun promoter DNA (Fig. 5A). This reveals over 30 protein spots capable of binding this DNA at this concentration (2 nM). After stripping (Fig. 5B), the signal is removed. The blot was then probed with a 19 bp duplex oligonucleotide containing the SMAD3/SMAD4 response element (representing +70 to +83 of this promoter [12]) (Fig. 5C) or the 20 bp MEF2 duplex oligonucleotide (representing −60 to −51 of this promoter). Neglecting the spots at the alkaline extreme of pH where resolution in the pI dimension is poor, there are only two protein spots labeled by SMAD3/SMAD4 while the MEF2 oligonucleotide shows a more complex pattern. Each of these spots was

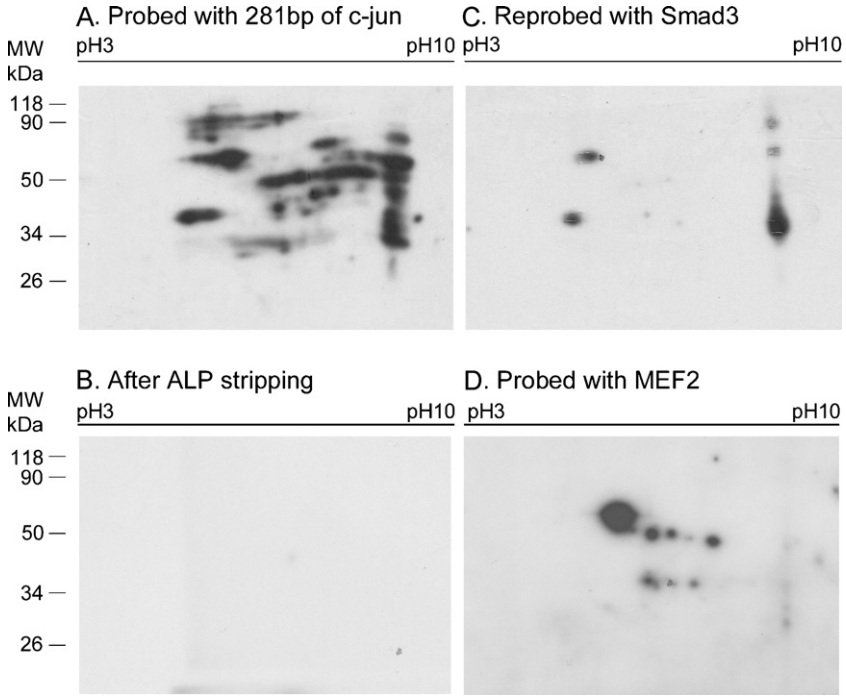

Fig. 5. Identifying a transcription factor by 2DGE-SW coupled with ALP stripping. The 2DGE-Southwestern blot was performed on 115 μg of HEK292 nuclear extract using an IEF strip with pH range from 3 to 10, followed by a second dimension of SDS-PAGE on a 10% gel. After electroblotting, the PVDF membrane was probed with: (A) radiolabeled 281 bp c-jun promoter (2 nM) to show all proteins which can bind this promoter. (B) The signals are removed by ALP stripping. Reprobing with 5 nM radiolabeled SMAD 3 (C) or MEF2 (D) shows the spots identified by these oligonucleotide probes.

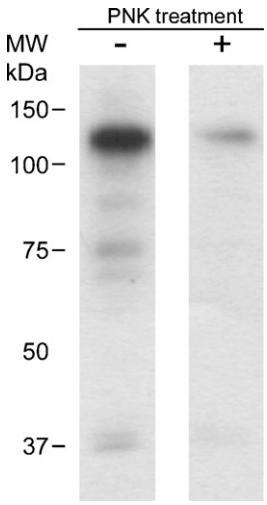

Fig. 6. T4-polynucleotide kinase stripping. Nuclear extract (10 μg) was applied to SDS-PAGE, blotted and blocked in SW buffer (10 mM HEPES, pH 7.5, 10 mM $MgCl_2$, 50 mM NaCl, 0.1 mM EDTA, 1 mM DTT, and 0.1% Tween-20) containing 5% non-fat dried-milk overnight at 4 °C. The blot was probed in 2 ml of SW buffer containing 2 nM γ-^{32}P-ATP labeled Jun/Ku oligonucleotide, 5 μg of poly (dI:dC) and 2.5% of BSA at 4 °C overnight and exposed to film at −85 °C overnight to visualize DNA–protein binding, shown to the left. On the right, this blot was then incubated in 2 ml of SW buffer containing 15 units of T4-polynucleotide kinase (T4PNK, from New England Biolabs, Ipswich, MA, USA) catalogue number M201S, 10 units/μl), 200 μM of ADP and 2.5% of BSA at 37 °C for 30 min. After exposure overnight at −85 °C, the blot was again autographed. Most bands were completely stripped of signal except one faint band at the top.

also labeled by the intact 281 bp c-jun promoter. The identity of these spots is currently under investigation.

3.6. Polynucleotide kinase stripping

T4 polynucleotide kinase (T4PNK) catalyzes a reversible reaction in which a phosphate is transferred from ATP to 5′-OH-DNA in one direction or transfers a phosphate from 5′-phospho-DNA to ADP in the reverse direction. Both the forward and reverse direction reactions are commonly used in molecular biology [13]. In Fig. 6 is shown that the reverse T4PNK reaction provides an alternative form of stripping. The enzyme effectively removes the label from the probe DNA, though not as thoroughly as does lower amounts of alkaline phosphatase (Fig. 1). Because of the relative cost of the two enzymes and the efficiency at removing the probe signal, alkaline phosphatase stripping is recommended for most purposes. However, if alkaline phosphatase were found to affect the phosphorylation state of a particular transcription factor, T4PNK, which is specific for DNA, might provide a useful alternative.

4. Conclusions

Stripping of Southwestern blots has proved problematic. Procedures effective for other blotting techniques such as Western blots have proved unsuccessful for Southwestern blots and the only previous method we had found to work was SDS stripping [1], which clearly is of limited repetitive use (Fig. 2). The problem appears to be finding conditions that are harsh enough to remove the probe signal without irreversibly denaturing the blotted proteins or extracting them from the blot. The enzymatic approach used here shows great promise and would allow at least six-fold repeated probings on the same blot (Fig. 2) and with different DNA sequences (Figs. 3 and 5). It has minimal effect on overall protein phosphorylation under the conditions described although there can be no assurance that an individual protein will be unaffected. Stripping with T4 polynucleotide kinase and ADP worked well (Fig. 6) and would be costly to perform routinely but this enzyme should leave protein phosphorylations unaffected and could provide an alternative. Application to 2DGE Southwestern blots is also clearly feasible and useful (Fig. 5).

Acknowledgements

We appreciate the excellent technical assistance of Ms. Maria Macias and the comments of our labmates. This work was funded by the National Institutes of Health, Grant 5R01GM043609.

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