Repeated Probing of Western Blots Obtained from Coomassie Brilliant Blue-Stained or Unstained Polyacrylamide Gels

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For several reasons, it can be desirable to probe a filter obtained after Western blotting of polyacrylamide gels with different antibodies. On the one hand, protein amounts may be limiting and re-probing of a protein filter may be the only way to identify different antigens. On the other hand, in the case of two-dimensional (2-D) gel electrophoresis, exact reproducibility of the protein pattern is difficult, and reusing the same blot ensures reliable comparison among different polypeptides using the appropiate antibodies.

We have developed a method to reprobe polyvinylidene fluoride (PVDF) membrane blots obtained by electrophoretic transfer of proteins separated by either one-dimensional (1-D) sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) or by 2-D gel electrophoresis (isoelectric focusing [IEF], SDS-PAGE) up to 5 times. For detection of the antibody complexes, horseradish peroxidase-coupled secondary antibody and the enhanced chemiluminescence (ECL™) detection system from Amersham International (Braunschweig, Germany) were used.

Four of the five antibodies used for this study were raised in rabbits, and one was raised in mouse. The first antibody was raised against maize *rpoA* plastid gene product over-expressed in *E. coli*. This protein is a 38-kDa subunit of the soluble RNA polymerase encoded on the plastid DNA of higher plants. Two more antibodies raised in rabbit are specific for the α- and the β-subunit of plastid ATPase purified from spinach plastids (3). Their molecular weights are 55 and 57 kDa, respectively. The fourth antibody was raised against a synthetic polypeptide homologous to a 158-kDa protein purified from barley plastids. It reacts with both a 158- and a 154-kDa protein. Depending on the age of the plastids, one or two bands can be detected. An anti-α-tubulin antibody was purchased from Sigma Chemical (Munich, Germany). This antibody, which was raised in mouse, reacts with a 50-kDa protein.

Protein samples analyzed were derived from two types of barley plastids: mature chloroplasts or young chloroplasts enriched in proplastids ("proplastids"). Isolated intact plastids were disrupted in 50 mM Tris-HCl, pH 7.9, 10 mM $MgCl_2$, 25% (wt/vol) sucrose, 50% (vol/vol) glycerol, 0.2 M ammonium sulfate and 2 mM dithiothreitol (DTT) using a glass homogenizer. Centrifugation at 180 000× *g* for 3 h at 4°C yielded two fractions: a soluble fraction and a membrane fraction. The membrane fraction was used to prepare a transcriptionally active lysate according to Rushlow et al. (7).

The soluble protein fraction was purified by diethylaminoethyl (DEAE) anion-exchange chromatography (2). Protein-containing fractions were collected, combined and further purified by heparin-sepharose affinity chromatography (5). Fractions containing soluble RNA polymerase activity were collected and used for gel electrophoretic analysis.

Fractions of membrane or soluble proteins were boiled in SDS sample buffer and separated on a 7.5% SDS-PAGE (4). Two identical gels were run in a twin gel chamber (Biotec-Fischer, Reiskirchen, Germany) overnight at 16 mA. Proteins on one of the gels were silver stained (1). The second gel was equilibrated in 20 mM Tris, 150 mM glycine and 0.1% (wt/vol) SDS (cathode buffer) for 15 min and then trans-

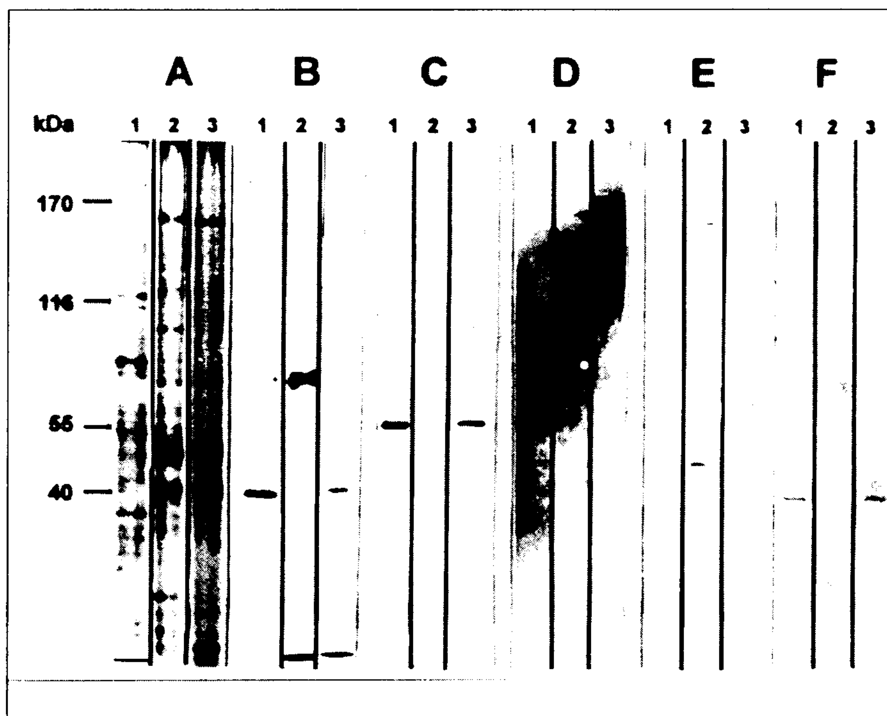

Figure 1. Silver-stained gel and Western analysis of a similar gel. (A) Transcriptionally active fractions derived from soluble barley chloroplast proteins (lane 1), from membrane-bound "proplastid" proteins (lane 2) and from membrane-bound chloroplast proteins (lane 3) were subjected to SDS-PAGE and silver stained. (B) Proteins separated on a similar gel as in A were transferred onto a PVDF membrane and treated with an antiserum raised against the *rpoA* gene product diluted 1:750 in TBST followed by incubation with horseradish peroxidase conjugated anti-rabbit IgG (1:1000 dilution). Proteins reacting with antibodies specific for the *rpoA* gene product were detected by the ECL detection system. (C) After erasing antibodies with NaOH, as described, the same blot was incubated with antiserum specific for plastid α-ATPase (1:2000 dilution in TBST) and detected as in B. (D) The same blot was subjected again to the erasing procedure and incubated with an antibody directed against a synthetic polypeptide homologous to a 158-kDa protein (1:600 dilution) purified from barley plastids. (E) The same blot was erased a third time, incubated with α-tubulin (1:500 dilution) antiserum and developed using peroxidase conjugated anti-mouse IgG. (F) After erasing antibodies a fourth time, the blot was incubated again with the antiserum specific for the *rpoA* gene as in B.

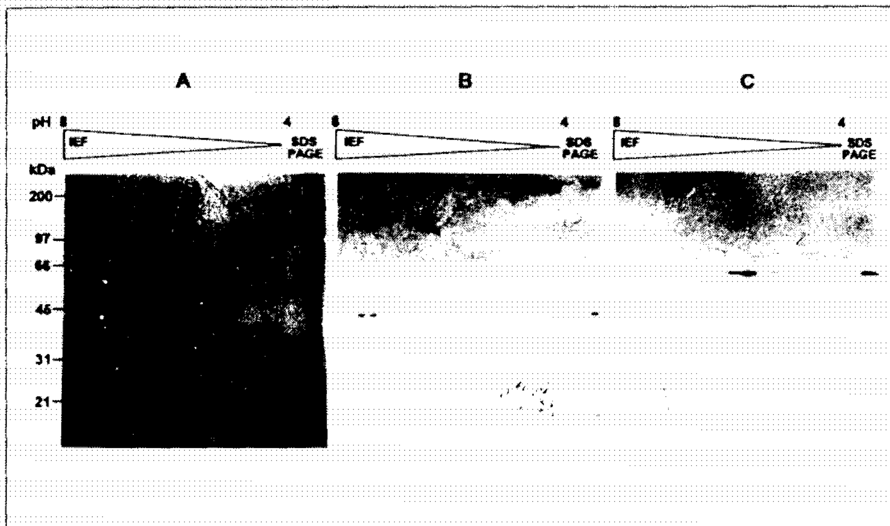

Figure 2. Immunodetection of proteins separated by 2-D gel electrophoresis, stained with Coomassie Brilliant Blue R-250 and transferred onto a PVDF membrane. (A) A membrane-bound transcriptionally active protein fraction from chloroplasts was subjected to IEF, subsequently separated by SDS-PAGE and stained with Coomassie Brilliant Blue R-250. (B) The same gel was blotted onto a PVDF membrane as described and incubated with an antiserum raised against the *rpoA* gene product. (C) After erasing the antibodies with NaOH, the same blot was incubated with β-ATPase antiserum diluted 1:2000 in TBST.

ferred onto two layers of Whatman 3MM paper (Whatman, Maidstone, England, UK), which had been soaked in the same buffer. A PVDF membrane (Immobilon™; Millipore, Bedford, MA, USA) was cut to the size of the gel, soaked in methanol for 1 s, rinsed in water for 2 min and equilibrated with 20 mM Tris, 150 mM glycine and 30% (vol/vol) methanol (anode buffer). The gel was covered with the pretreated membrane and with two layers of Whatman 3MM paper soaked in anode buffer. Blotting was carried out at 0.7 mA/cm^2 for 1.5 h at room temperature using a semi-dry blotting apparatus (Biotec-Fischer).

For 2-D gel electrophoresis, membrane-bound proteins from chloroplasts were dissolved in TAROT buffer [50 mM Tris-HCl, pH 7.6, 17.5% (vol/vol) glycerol, 10 mM ammonium sulfate, 4 mM EDTA, 2 mM DTT] before sample buffer [8.0 M urea, 0.4% (vol/vol)

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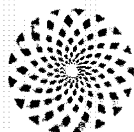

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For re-probing, antibodies were erased from the PVDF membrane by wetting the membrane in tap water and incubating it further for 5 min in 0.2 M NaOH. Blots were then rinsed in water, incubated in TBST, blocked and exposed to antibodies as described before.

Two examples shown here demonstrate the advantages of the method. The blot obtained by transfer of proteins from an SDS-containing polyacrylamide gel was first immuno-decorated with the anti-*rpoA* antibody (Figure 1B). A 38-kDa band corresponding to the known size of the *rpoA* gene product was detected in the soluble as well as in the membrane protein fraction of chloroplasts (Figure 1B, lanes 1 and 3). In addition, a cross-reactive band of 68 kDa was detected in the membrane fraction of proplastids (Figure 1B, lane 2). The blot was erased with NaOH as described and incubated with the antibody specific for the α-ATPase subunit. None of the previously visible bands was detectable. Instead, a 57-kDa band corresponding to the known size of the α-subunit of plastid ATPase was visible in both the soluble and membrane fractions from chloroplasts (Figure 1C, lanes 1 and 3). No signal was obtained in the membrane fraction from the "proplastids" (Figure 1C, lane 2). The blot was erased again and incubated with an antibody raised against a synthetic peptide homologous to a 158-kDa protein isolated from the membrane protein fraction of chloroplasts. This antibody detected two proteins of 158- and 154-kDa molecular weight, respectively, in the membrane protein fraction of chloroplasts and a single band of 158 kDa in the membrane protein fraction of "proplastids" (Figure 1D, lanes 2 and 3). No signal was detectable in the soluble fraction of chloroplasts (Figure 1D, lane 1). Again, the immunoreactive bands detected by the previous antibody were not visible. The blot was erased a third time and incubated with the fourth antibody, which is specific for α-tubulin. This antibody reacted with a 50-kDa protein in the membrane fraction of proplastids (Figure 1E, lane

2). As in the case of the other immunoreactions, no cross-reactivity from the previous antibody bindings was carried over. To test whether the *rpoA* protein could be detected again after erasing the blot several times, the erased blot was incubated with the same antibody as used in the first round of immuno-decoration. The 38-kDa protein could be detected in the soluble and membrane fraction of chloroplasts as before (Figure 1B, lanes 1 and 3; Figure 1F, lanes 1 and 3). This indicates that repeated immuno-decoration of the same blot neither impairs the specificity of the antibody binding nor leads to the full loss of proteins from the PVDF membrane. However, the signals are weaker, which may be due either to a partial loss of protein from the PVDF membrane or to destruction or reduced accessibility of the epitope. In addition, the nonspecific background increased during repeated re-probing of the same membrane filter.

The same re-probing procedure was used for a membrane carrying proteins separated by 2-D gel electrophoresis (Figure 2). In this case, the gel was first stained with Coomassie Brilliant Blue R-250 (Figure 2A) and was then blotted onto a PVDF membrane. Immuno-decoration with the antibody specific for the *rpoA* gene product revealed several spots representing proteins of 38-kDa molecular weight (Figure 2B), which have different isoelectric points. This blot was erased and re-probed as described with an antiserum specific for the β-subunit of plastid ATPase. After this treatment, the previously detected 38-kDa protein was no longer visible. Instead, a protein spot corresponding to the known size of the 55-kDa β-subunit of ATPase was detected (Figure 2C).

Staining and repeated immuno-decoration of the same blot after 2-D gel electrophoresis ensures reliable comparison among protein patterns and cross-reactivities. 2-D PAGE is a long and laborious procedure with limited reproducibility. Moreover, we have shown that the same 2-D gel can be stained with Coomassie Brilliant Blue R-250 to reveal the overall protein pattern and, subsequently, can be blotted and repeatedly probed using different antibodies to reveal the presence and location of specific antigens.

Compared to an SDS-based method described for re-probing Western blots (8), our method requires only five minutes for stripping and only one chemical. It can be performed at room temperature and has been shown to work also for 2-D gels. In addition, our method can be used on Coomassie Brilliant Blue-stained as well as unstained gels.

REFERENCES

1. **Blum, H., H. Beier and H. Gross.** 1987. Improved silver staining of plant proteins, RNA in polyacrylamide gels. Electrophoresis *8*:93-99.
2. **Gruissem, W., B.M. Greenberg, G. Zuraw-**

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ski and R.B. Hallick. 1986. Chloroplast gene expression and promoter identification in chloroplast extracts, p. 253-270. *In* Methods in Enzymology, Vol. 118. Academic Press, New York.

3. **Herrmann, R.G., P. Westhoff, J. Alt, J. Tittgen and N. Nelson.** 1985. Thylakoid membrane proteins and their genes, p. 233-256. *In* L. van Vloten-Doting, G.S.P. Groot and T.C. Hall (Eds.), Molecular Form and Function of the Plant Genome. Plenum, New York.
4. **Laemmli, U.K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature *227*:680-685.
5. **Lerbs-Mache, S.** 1993. The 110-kDa polypeptide of spinach plastid DNA-dependent RNA polymerase: single-subunit enzyme or catalytic core of multimeric enzyme complexes? Proc. Natl. Acad. Sci. USA *90*:5509-5513.
6. **Rickwood, D., J.A.A. Chambers and S.P. Spragg.** 1990. Two-dimensional gel electrophoresis, p. 217-272. *In* B.D. Hames and D. Rickwood (Eds.), Gel Electrophoresis of Proteins. Oxford University Press, New York.
7. **Rushlow, K.E. and R.B. Hallick.** 1982. The isolation and purification of a transcriptionally active chromosome from chloroplasts of *Euglena gracilis*, p. 543-550. *In* M. Edelman, R.B. Hallick, N.-H. Chua (Eds.), Methods in Chloroplast Molecular Biology. Elsevier Biomedical Press, Amsterdam.
8. **Tesfaigzi, J., W. Smith-Harrison and D.M. Carlson.** 1994. A simple method for reusing Western blots on PVDF membranes. BioTechniques *17*:268-269.

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Use of ^{33}P for Ribozyme Assays: The Safe Way

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Ribozymes or RNA enzymes are promising tools for the specific inhibition of gene expression and virus replication in animal or plant cells. Studies of the in vitro cleavage of synthetic radiolabeled RNA target by labeled or unlabeled ribozymes is a necessary step before in vivo applications. These cleavage assays are usually performed using ^{32}P-labeled transcripts, which imposes the handling of unnecessary large quantities of high-energy radioactivity. The transcripts are not used in excess as probes that have to hybridize to nonradioactive nucleic acids fixed to a membrane, but they are all part of the reaction and detected directly in the dried gel.

To reduce the exposure to high levels of radioactivity, different methods have been tested. Recently, ribozyme self-cleavage assays using nonradioactive labeled RNA was demonstrated (5). Depending on the constructs tested, steric hindrance due to the haptene-labeled nucleotides may prevent or reduce the interaction with the target and

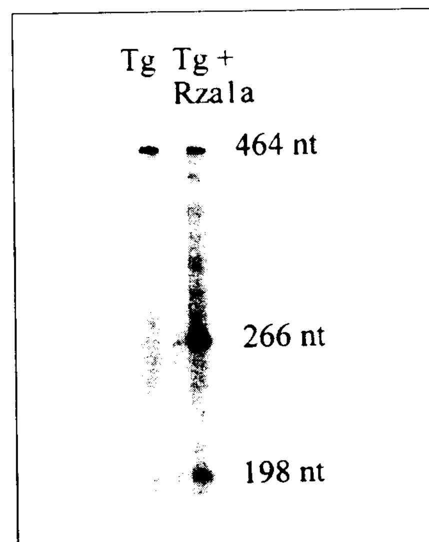

Figure 1. Cleavage of the ^{33}P-labeled 464-nt RNA target by the nonradioactive 243-nt ribozyme. The two transcription products were incubated 90 min at 60°C in 50 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$. Half of the reaction was denatured for 1 min at 95°C and loaded on a 6% acrylamide gel.