Strategy for stripping Western blots and reprobing with ECL Western blotting

Introduction

Enhanced chemiluminescence has become the most commonly used method for detecting proteins after Western blotting. One of the many advantages it has over colorimetric detection methods is the ability to re-use blots after initial immunodetection and thus obtain further information. A previous application note (1), following the method of Kaufman *et al* (2), outlined a successful protocol for stripping and reprobing of Western blots. Data (not shown) indicates that in excess of 50% of some target proteins can be lost when performing experiments where PVDF and nitrocellulose blots are stripped and reprobed. It is therefore important to consider which antigen is present in least abundance and probe for this first. The following article gives an example where three antibodies have been detected, stripped and redetected with ECL™ Western Blotting Detection Reagents. A suggested strategy for stripping and re-detecting in four different situations is also included.

Warning

Safety procedures as set out in the product literature should be observed when using the products described here.

Method

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Separation and immunodetection

1.1 Rat brain homogenate was separated using 10% SDS-PAGE and blotted onto Hybond™ P.

1.2 The membrane was blocked with 5% ECL Blocking Agent (RPN2125) in PBS containing 0.1% Tween™ 20 (PBS-T) for 60 min.

1.3 The membrane was rinsed in two changes of PBS-T.

1.4 The membrane was incubated in a mixture of 1:1000 anti-actin, 1:500 anti-160 kD neurofilament, and 1:500 anti-68 kD neurofilament antibodies diluted in PBS-T for 60 min.

1.5 The membrane was washed 3 × 10 min in PBS-T.

1.6 The membrane was incubated in 1:5000 anti-mouse IgG HRP conjugate (NA931), diluted in PBS-T for 60 min.

1.7 Wash step 1.5 was repeated.

1.8 The membrane was detected with ECL Western Blotting Detection Reagents and exposed to Hyperfilm™ ECL for 1 min.

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Stripping

After the first detection the membrane was washed as in step 1.5 and submerged in stripping buffer (100 mM β-mercaptoethanol, 2% sodium dodecyl sulphate, 62.5 mM Tris-HCl, pH 6.7) at 60 °C for 30 min with occasional agitation. (60 °C was determined as optimum incubation temperature for these three antibodies.)

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Confirmation of stripping

3.1 The membrane was washed for 2 × 10 min in PBS-T using large volumes of buffer (4 ml/cm^2).

3.2 The membrane was blocked by immersing in 5% ECL Blocking Agent dissolved in PBS-T for 60 min.

3.3 To determine if any primary antibody remained the membrane was rinsed in two changes of PBS-T and steps 1.6-1.8 were repeated.

3.4 The method was repeated twice starting from step 1.2. On the final occasion the method was followed until step 1.8.

Results

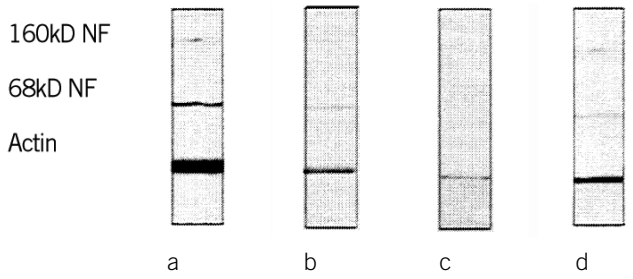

Fig 1. Rat brain homogenate Western blots showing initial immunodetection and detection following stripping procedures.
a – Simultaneous detection of three antigens directly after blotting. 1 min exposure to film
b – Repeat detection after a stripping procedure. 1 min exposure to film
c – Second repeat detection after two stripping procedures. 1 min exposure to film
d – A repeat of Figure 1c exposed to film for 5 min.

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Discussion

The initial temperature optimization strategy shown in Figure 2 is universal, less time is wasted if the stripping conditions are optimized using dot blots before Western blots are prepared. However this can only be done when sufficient pure protein is available. To obtain the best results when dot blots cannot be prepared it is suggested that the incubation temperature for stripping is started at 50 °C and the stripping efficiency checked using the confirmation of stripping protocol. If this is incomplete the stripping may be repeated at 5 °C temperature increments. In this experiment no target antigen was visible after stripping when the membrane was processed according to the confirmation of stripping protocol and exposed to film for 10 min (results not shown).

Loss of target from the membrane for some proteins may be less than the 50% already mentioned. The strategies outlined in this article are intended as a guide only to help in obtaining the best possible results.

Four strategies are outlined in Figure 3. In the first strategy, if both the antibody affinities and the abundance of each antigen are approximately equal then it is not important which antigen is detected first.

The second strategy highlights the importance of detecting antigens of similar abundance with the lowest affinity antibody initially, so that less vigorous stripping is performed first, ensuring the greatest chance of detecting both antigens before and after stripping.

The results shown in Figure 1 demonstrate the importance of the third strategy, which suggests detecting the lowest abundance antigens first if the primary antibodies used are of equal affinity. After the initial detection, all three antigens are visible (see Fig 1a), after stripping and reprobing it is possible to detect both 68 kD neurofilament and actin but is difficult to see the 160 kD neurofilament band (see Fig 1b). After a further stripping and reprobing only the actin band is visible (see Fig 1c). This shows that if these antigens are to be detected sequentially the 160 kD neurofilament should be detected first then the 68 kD neurofilament and finally actin. If a band is no longer visible it may be possible to detect the band by extending the exposure time to autoradiographic film. The exposure times to film for Figures 1a, 1b and 1c was 1 min. Enhanced chemiluminescence is highly sensitive and extending the exposure time of Figure 1c to 5 min (see Fig 1d) allowed visualization of all protein bands.

The fourth strategy is harder to evaluate but in general it is best to detect the antigen of lowest abundance first rather than use the lowest affinity antibody. This is because target loss is likely to have the greatest effect on the ability to detect antigen.

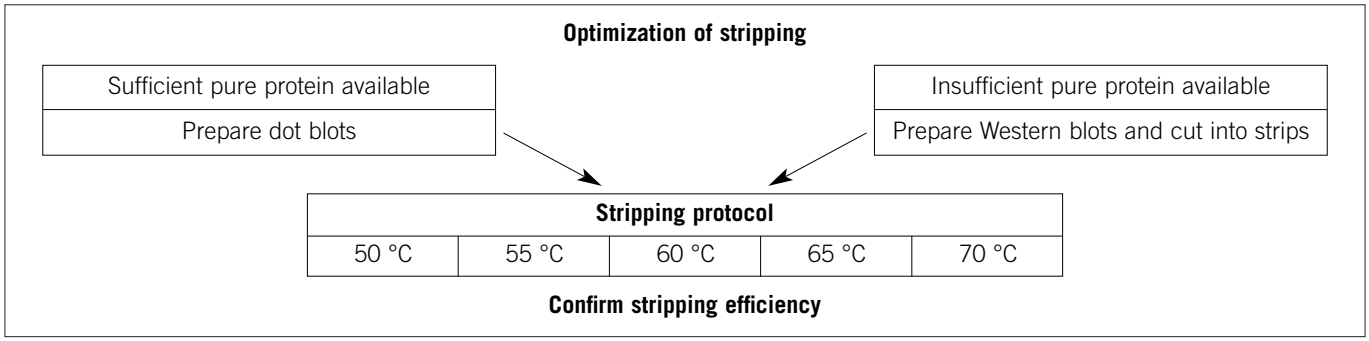

Fig 2. Initial temperature optimization for stripping

Strategy 1	Strategy 2	Strategy 3	Strategy 4
Two antigens of similar abundance and two antibodies of similar affinity	Two antigens of similar abundance, antibodies of unequal affinity	Two antigens, one high and the other low abundance, antibodies of equal affinity	Two antigens, one high and the other low abundance, antibodies of unequal affinity
Detect either antigen first	Detect either antigen with lowest affinity antibody first	Detect low abundance antigen first	Detect low abundance antigen first
Strip	Strip	Strip	Strip
Detect remaining antigen	Detect antigen with highest affinity antibody	Detect high abundance antigen	Detect high abundance antigen

Fig 3. Approach to determine optimum stripping strategy

Products used

Amersham Pharmacia Biotech products used for this process:

Hybond P (20 × 20 cm)	RPN2020F
Hyperfilm ECL (18 × 24 cm)	RPN2103K
Anti-mouse IgG HRP conjugate	NA931
ECL Western Blotting Detection Reagents	RPN2106

Other materials required

Tween 20

References

1. Application Note: *Stripping Western Blots for Reprobing with Enhanced Chemiluminescence (ECL).*

2. KAUFMAN, S.H., EWING, C.M. and SHAPER, J.H., Analytical Biochemistry, *The Erasable Western Blot*, **161**, pp.89.95 (1987).

Product information

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