Sample preparation by SDS-PAGE and electroblotting onto PVDF for Protein Sequencing

In order to get the best results out of an N-terminal sequence analysis of samples prepared by SDS-PAGE and electro-blotting onto PVDF (polyvinylidene difluoride), the following considerations and do's and don'ts are given below.
Note. *N-terminal as well as internal sequence(s) of proteins purified by SDS-PAGE can also be obtained without blotting (see our brochure for these services).*

General remarks
Standard Laemmli [1] or Tris-Tricine [2] gels and blotting procedures can be used, however, initial yields of sequence analysis may be as low as 5% of the original quantity of protein loaded onto the gel. If sufficient sample (100 pmol or more) is used, no special precautions are necessary. The do's and don'ts given below are especially of importance if low quantities of protein material should be sequenced. The most important precautions to raise the initial yield are listed first; minor improvements are given later.

To minimize N-terminal blocking
Age gels for at least 24 hours. Add thioglycolate (0.1 mM final) to electrophoresis buffer. Minimize chemical oxidation of amino acids by minimizing activators APS and TEMED. Prepare PAGE gels with the purest acryl amide available. Treat acryl amide stock with Amberlite (1 g/100ml). Preferentially use PDA (piperazine diacryl-amide, Biorad)[3] as crosslinker, and HPLC grade glycerol to minimize aldehyde formation.
Much higher initial yields of N-terminal amino acids are obtained if gel systems are used having a running gel at lower pH (e.g. pH 7.2 [4,5]) then in case of the Laemmli system [6]. Sometimes, pre-electrophoresis with 0.1 mM thioglycolic acid leads to higher initial yields as well [6,7].
Use minimal amount of glacial acetic acid when staining/destaining (max. 1%). If possible, incubate samples for 10 minutes at 37 ^{o}C in denaturation buffer instead of boiling for 3-5 minutes for possible better Initial Yields.

To minimize losses
Use mini-gels (10 x 10 cm x 0.5-0.75 mm) when only limited protein material (< 100 pmol) is available [8]. Concentrate as much as possible protein per area of PVDF. There are several kinds of PVDF commercial available (Immobilon Millipore/Waters, PVDF from Biorad, or ProBlott of Applied Biosystems). Problott offers high binding capacity, and is advised.

About blotting
Proteins should be blotted **as soon as possible** after electrophoresis, without fixation of the gel. Soak the gel for 5 minutes in transfer buffer prior to blotting. Meanwhile, pre-wet PVDF membrane with 100% methanol for a few seconds, and **immediately** transfer it to the blotting buffer used. Use two layers of PVDF (especially with Immobilon) for security.
Different transfer buffers can be used. Good results have been obtained with 10 mM CAPS pH 11 /10% methanol [9] or a 25 mM Tris-HCl/192 mM Glycine buffer pH 8.3 /15% methanol [10] as transfer buffer, but also with a borax buffer pH 8.25 /15% methanol. In case of proteins with unknown pI's, the first method is the one of choice (keep pH of transfer buffer above isoelectric point).
Time and power (V/A) to be used during electro-blotting depends on a) type of apparatus; b) the range of Mw's which are to be blotted; c) type of buffer; d) amount of SDS; and, therefore, should be tested and optimized. General conditions for 'tank' (wet) blotters and using CAPS buffer: for 0.5 x 10 x 10 cm gels, 50V (about 100-200 mA) for 30 minutes at room temperature; Tris-Glycine: 40V (300 mA), R.T. for 1-4 hours; for 'semi-dry' 1-2.5 mA/cm^2 for < 30 min.
Note! The electrophoresis conditions given in the article by P. Matsudaira [9] are not suitable (too fast; proteins remain in gel and in 2nd layer).

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If a 'tank-blotter' is used, it is advisable to use p.a. grade methanol for blotting buffers in order to minimize the quantity of aldehydes which might be present.

Staining

Wash the blot with distilled water prior to staining (3 times 1 minute) to decrease the amount of salts and buffer components. The electro-blotted proteins are bound very tight to the PVDF membrane and cannot be washed off with water. Detect proteins preferentially by staining with Coomassie Brilliant Blue (stain max. 5 min.) [9]. First saturate PVDF with 100% methanol for a few seconds. Destain (partially) with 50% methanol. A quick destaining can be performed by changing the destaining solution several times, and soaking for 1-2 minutes between changes. A further destaining can be achieved by washing with 90% propanol, however, complete destaining is not necessary for sequence analysis. Amido Black and Ponseau S can also be used as staining techniques.

Initial Yields; quantity of protein available for sequencing

Due to partial N-terminal blocking during electrophoresis, low overall **initial** yields (5-30%, particularly with the Laemmli system) are routinely found [6]. This should be taken into account when loading a sample on the gel. SDS-PAGE systems with a running gel pH of 7.2 give higher initial yields (40-60%)[6,5,4]. Signals on a level of 1 pmol (if pure) can be interpreted reliable, although a level of 2-10 pmol is preferred.
The foregoing means, that for an N-terminal sequence analysis of proteins blotted onto PVDF, at least 25 - 50 pmol of protein should be applied onto an SDS-gel; and at least 100 pmol of starting material should be used if internal sequences have to be determined in case of a blocked N-terminus; e.g. if you want to prepare samples by means of a CNBr cleavage or Cleveland digestion followed by SDS-PAGE and blotting [7,11].
For fast internal sequence analysis of proteins present in a piece of gel, Eurosequence offers a special service, which is suitable for 50 pmol quantities (or even less) of protein material. Just send us a piece of gel containing the protein of interest, and we'll take care of the rest.

Furthermore

The total area of the piece of PVDF containing the protein band should be as small as possible and should not exceed 0.8 cm^2. Try to concentrate as much as possible protein per area of PVDF.
We are able to sequence PVDF-blotted proteins almost as effective as proteins spotted on polybrene coated glass filters. Runs with more than 40 positively identified residues have been performed with 100 pmol of protein.

Literature

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