Blotting Techniques for the Detection of Protein Polymorphisms in Stains

S. Rand

Institut für Rechtsmedizin der Westf. Wilhelms-Universität, Münster, von Esmarch-Str. 86, D-4400 Münster, FRG

INTRODUCTION
The aim of this paper is to give a review of the methods available for the detection of protein polymorphisms in stains. The characteristics of the various blotting methods are described as a guide to selection of the most suitable for the protein under investigation. With this in mind the paper has been subtitled "The successful blot - luck or judgement?". Most problems, which occur are due to human error and can be avoided if proper care is taken. However to identify the cause of a particular problem background knowledge and experience is essential. The various stages involved are discussed here in detail.

IMMUNOCHEMOPHORESIS:
Blotting is only one of the essential stages in the complete process for detection of protein polymorphisms, which has come to be known as immunochemophoresis (Rand et al 1989). The individual stages are shown in Figure 1.

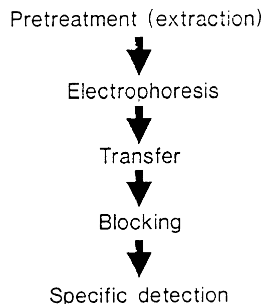

Fig.1: Stages involved in immunochemophoresis

Pretreatment:
During stain formation and/or storage proteins can undergo changes which then require some form of pretreatment before successful typing can be carried out. The pretreatment stage can be combined with the extraction of the protein from the stain. Two important factors are:
- Cleavage of sialic acid residues (e.g. N-acetylneuraminic acid: NANA) where the unequal loss of NANA moieties leads to inhomogeneities in the protein molecules. The action of neuraminidase strips the NANA-residues leaving only the protein backbone.
- The reductive cleavage of internal disulphide bonds of the protein which can be oxidised during stain formation to form complexes within the same molecule or with other proteins or fragments of proteins.

Pretreatment methods for some important proteins are listed in Rand (1990).

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Electrophoresis:

Separation of the protein subtypes can be carried out in a variety of gels, but the most commonly used are agarose and polyacrylamide. The modifications for each particular system are too numerous to list here and the reader is advised to read Spielmann and Kühnl (1982), Gaensslen (1983) and Rand (1990) for further details.

BLOTTING:

The blotting technique was first described by Southern (1975) for the transfer of DNA molecules and became known as "Southern Blotting". Subsequent modifications for use with other molecules were also geographically named. The term "Northern Blotting" was used for transfer of RNA (Alwine et al 1977), "Western Blotting" for proteins (Burnette 1981) and last but not least a variation called "Eastern Blotting" (Reinhart and Malamud 1982) also for proteins. There are 4 basic types of blotting mechanism.

Diffusion blotting:

This is the simplest form of blotting. The transfer relies solely on diffusion of the proteins from the gel to the membrane (Bowen et al 1980). Transfer can be carried out unidirectionally by placing a membrane directly on the gel without removing the gel from the glass backing plate. Filters paper are then placed on the membrane and covered by protective foil and small weight.

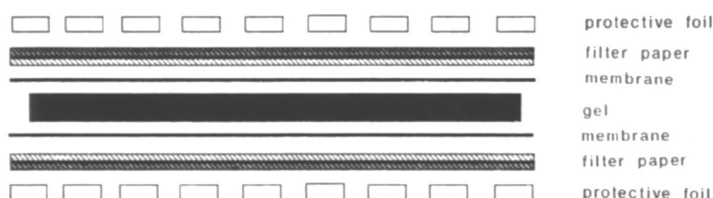

Fig.2: Construction of diffusion blotting

The method is simple but relatively inefficient (ca. 50% transfer) and many hours are required for adequate transfer. The advantage of this method lies in research in the form of bidirectional blotting whereby two identical copies can be obtained by placing a membrane on either side of the gel. However the disadvantages of this method do not make it suitable for stain work.

Capillary blotting:

Capillary blotting, first described by Southern (1975), is more efficient and less time consuming than simple diffusion blotting. Transfer of protein molecules is enhanced by the capillary flow of buffer which passes from the reservoir through the gel to the membrane carrying the molecules along the capillary gradient (Fig.3).

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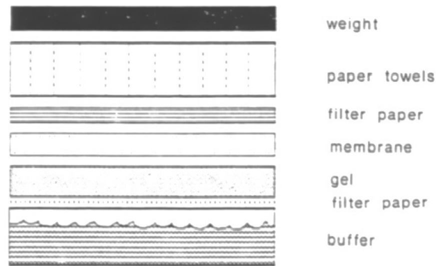

Fig.3: Construction of capillary blotting

Vacuum blotting:
In order to increase the flow of buffer through the gel to the membrane, vacuum blotting has been suggested as a more efficient alternative (Peferoen et al 1985). Although an increased flow rate is achieved the method has not found wide acceptance because the apparatus is more difficult to operate. The set up is essentially the same as capillary blotting except that a vacuum apparatus is placed on top of the membrane.

Electroblotting:
The concept of electroblotting was first introduced by Towbin et al (1979) and the protein molecules are transferred from the gel to the membrane due to a field gradient set up between 2 electrodes. There are 2 main versions of electroblotting. The first method described was vertical electroblotting (Fig. 4) in which the gel and transfer matrix (membrane) were placed in an electrophoresis tank between two electrodes and submerged in buffer.

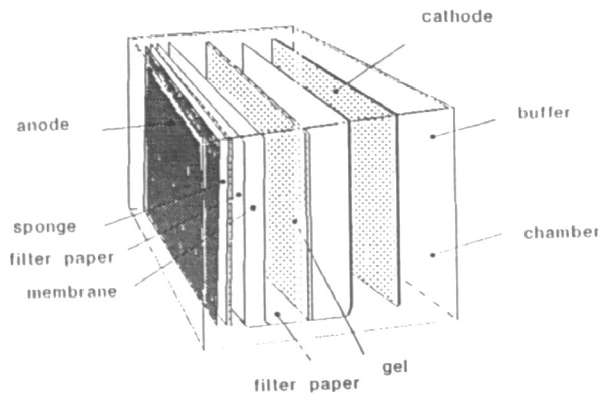

Fig.4: Construction of tank electroblotting

Due to problems of handling the gels had to be relatively stable and this method is not suitable for the more modern ultrathin polyacrylamide gels. A more recent innovation is shown in Fig. 6, the semi-dry electroblotting method. Blotting is carried out in a horizontal system using graphite electrodes (Kyhse-Andersen 1984). A "transunit pack" consists of filter

paper moistened with the anodic and cathodic buffers placed on either side of the gel and membrane (Fig.5).

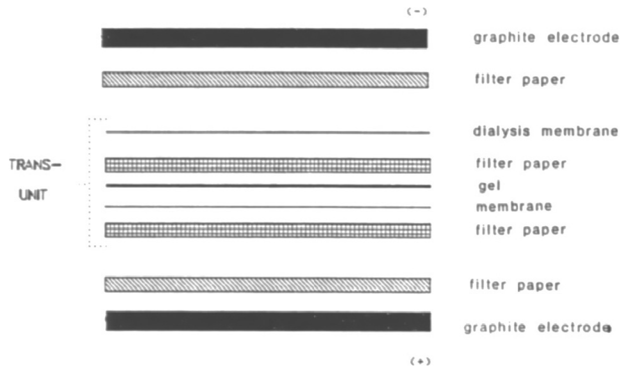

Fig.5: Construction of semi dry electroblot

Transfer is extremely efficient and vitually 100% transfer can be achieved within one hour. The method is also suitable for ultrathin polyacrylamide gels. By placing the membrane directly on the electrophoresis gel and topped by a light weight the gel can then be carefully pealed away from the glass backing plate together with the membrane and placed on the filter paper which has been soaked in the appropriate buffer. The use of block graphite electrodes also ensures the homogenous transfer of proteins across the whole of the gel. Many transunit packs can also be assembled together enabling multiple transfer to be carried out simultaneously. This is by far the most efficient method for transfer of proteins and has also become the most widely used.

Dot blot:
In this method the substance under investigation is spotted directly onto the membrane. It is suitable for use with soluble blood group substances or DNA typing but not for the detection of protein subtypes. The subsequent stages involved are however similar to those described here.

Advantages of blotting:
The new generation of detection methods are multistage procedures involving extensive washing and incubation stages. It is therefore necessary to transfer the electrophoretically separated proteins from the gels to a stable and flexible matrix (membrane). This allows ease of handling and provides a permanent record of the results. Because the proteins are distributed throughout the thickness of the gel the transfer to the surface of a membrane will cause a concentration effect and an increase in sensitivity. Multiple probings with different detection techniques can be carried out on one membrane either by stripping the previous antibody from the membrane or by subsequent incubation using different antibody-substrate combinations (Steffen and Linck 1989). While multiple immunoblots are theoretically possible there are few systems in the forensic repertoire which can be combined giving optimal separation for each system and this is not generally recommended for stain work.

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Because of the increase in sensitivity the antibody solutions can be used in a very diluted form (e.g. 1:500 or 1:1000) resulting in a substantial reduction in cost (for details see Rand 1990).

Blotting efficiency:

The efficiency of the blotting procedure is dependent on a variety of factors. The most important ones are listed below:

Gel: - Agarose gel has large pore sizes which allow freedom of flow for all molecules independent of their size and can be used in combination with diffusion blotting. The very small pore sizes in polyacrylamide gels lead to unequal and therefore selective transfer of different protein bands when relying only on diffsion blotting. An active mechanism of transfer such as capillary blotting or the much more efficient electroblotting is not subject to such limiting factors and both are suitable for use with polyacrylamide gels.

Membrane: - The binding capacity of the transfer matrix (membrane) and the mechanism of binding play a very important role in blotting. The appropriate membrane must be selected depending on the characteristics of the protein under investigation (Table I).

Membranes	Binding mechanism	Efficiency ($\mu g/cm^2$)
Nitrocellulose	hydrophobic	80
Nylon	electrostatic	480
DBM/DBT	covalent	10-20
DEAE	ionic	115
Teflon		
Polybrene		
Activated glass fibre		
CNBr		

Table I: Membranes used for protein blotting

The type of transfer apparatus used is not the only important criteria for achieving optimal transfer. Another important factor is the elution efficiency of the particular protein from the gel to the membrane. This is mainly influenced by the acrylamide concentration, the degree of crosslinking in the gel, the ionic strength and pH of the buffers used. The addition of other reagents to the gel or transfer buffer may also affect the quality of transfer. Methanol for example decreases the elution efficiency because the proteins will - to some extent - be denatured but increases the binding capacity of nitrocellulose membranes. The inclusion of SDS and urea in gels necessitates the use of alternative blotting conditions (for details see reviews by Beisiegel 1986; Gershoni and Palade 1983). The size and charge of a particular protein molecule will also play an important role. Large proteins - ie greater than 100 000 Daltons - are particularly susceptible to the factors described above but as most of the proteins investigated in forensic work are smaller this is usually not a great problem.

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Blocking:

In order to prevent non-specific binding of antibodies to the membrane it is necessary to block the unoccupied binding sites after blotting (quenching) using a neutral substance. The blocking agent used depends on the type of membrane and the protein under investigation. There is no general rule as to which should be used for which protein and the various alternatives must be tested for each particular system. Some of the more common blocking agents are listed in Table II. Factors which must be borne in mind when selecting a blocking agent are:

- No crossreaction should occur with any of the antibodies used in subsequent stages.
- Care must also be taken when using non-ionic detergents because they can cause dissociation and subsequent elution of proteins from nitrocellulose membranes (Stott 1989).

	Reagent	Concentration	
-	BSA	5	%
-	Tween 20	0.05	%
-	Gelatine	0.25	%
-	Serum	10	%
-	Casein	1	%
-	Bactogelatin	0.1	%

Table II: List of some common blocking reagents

DETECTION METHODS:

A variety of methods are available for the localization and visualization of proteins once they have been fixed on the membrane, but can be divided into 3 categories:

Unspecific Staining:

General protein stains such as Coomassie Blue or Amido Black can be used to localize proteins. Although this is the most simple method it is also the most insensitive, is non-specific and it can sometimes be difficult to identify the protein in question. The sensitivity and specificity can be improved by including incubation with a specific antibody by immunofixation. The so called immunofixation is still relatively insensitive. The use of silver staining (see Carracedo et al 1983) greatly improves sensitivity but the lack of specificity and high background are disadvantageous.

Direct labeling:

Specific labeling of the protein can be achieved by incubating the membrane with a specific antibody to which a label or enzyme has been incorporated. However the antibody will bind to the antigen site on a one-to-one basis so that although specificity has been achieved sensitivity depends on the amount of label. The most common enzyme/label combinations are listed in Table III.

Indirect labeling:

For this method the first (primary) antibody is directed against the specific ligand or antigen. The membrane is then incubated with a secondary labeled antibody directed against the Fc part of the primary antibody. Alternatively a third bridging antibody can be incorporated in the reaction between the primary and secondary antibodies as was initially proposed by Steinberger et al (1970).

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- Radiolabeling
- Biotin-Avidin
- Biotin-Streptavidin
- Alk. phosphatase
- Peroxidase
- Immunogold + Silver enhancer
- Chemiluminescense

Tab.III: Common labels and enzymes used for detection of proteins

By the use of indirect labeling the sensitivitiy of the method can be increased multifold because the ratio of antigen sites to label is no longer on a one-to-one basis.

Many labeling methods can be used to detect proteins. The most common detection systems are listed here:

Radiolabeling:
This has not found wide acceptance for protein blotting mainly due to precautions which are neccessary for handling radioactivity. No great advantage in sensitivity is observed and relatively long exposure times are necessary.

Enzyme conjugated probes:
Enzyme conjugated antibodies are the most commonly used method for detection of proteins. The enzyme must be subsequently incubated with a chromogenic substrate which is converted to an insoluble coloured precipitate at the site of activity. The 2 enzymes in common use are horseradish peroxidase and alkaline phosphatase which can be combined with a wide variety of substrates; the main ones are listed in Table IV.

Peroxidase	- 4-Chloro-1-naphthol
	- Diaminobenzidine
	- o-Dianisidin
	- Luminol/luciferin
	- Dioxetane
	- 3-Amino-9-ethyl carbazole
Alk. phosphatase	- 5-Bromo-4-chloro-3-indolylphosphate
	- Nitro blue tetrazolium
	- ß-Naphthyl phosphate

Tab.IV: Possible combinations of enzyme/substrate labeling systems

There are two modifications of this method which should be mentioned.
As an alternative to the chromogenic substrate it is possible to combine the peroxidase-conjugated antibody with luminol. The substrate is oxidised to a chemiluminescent product which reacts in the form of emission of light photons, and can then be detected by exposing the membrane to an X-ray film. This method has only recently been developed but would appear to offer many advantages including increased sensitivity, easier handling and extremely short incubation times.

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The second alternative is the inclusion of a biotin/avidin or streptavidin complex as a bridging molecule. The most common method is the use of a biotinylated secondary antibody and avidin or streptavidin complexed with a biotinylated enzyme. This method exploits the very high affinity of biotin for multiple binding sites avidin and streptavidin and leads to a "christmas tree" effect and a multifold increase in senstivity.
The labeling of antibodies with colloidal gold with or without the addition of a silver staining method is claimed to be very sensitive. As little as 50 pg of antigenic protein can be detected but as yet this method has not been widely used for protein detection.
Flurescence labeling of antibodies has also been proposed but has not found wide acceptance probably due to its relative insensitivity. A more detailed review of the various methods is given by Stott (1989).

SUMMARY

Protein blotting has become a commonly used application in the repertoire of forensic stain investigations. A variety of methods including different stain extraction procedures, protein pretreatments, blotting techniques and detection methods have been developed, so that the user must select the optimal and most suitable method for detecting each particular protein.

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