CRC Handbook of Immunoblotting of Proteins

Volume I
Technical Descriptions

Ole J. Bjerrum

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PREFACE

The development of the immunoblotting technique has been an event of major importance in protein chemistry. The combination of the high resolving powers of electrophoretic separation techniques with the specificity of antibody detection has brought a new dimension into the molecular characterization of proteins.

In spite of a landslide of methodological papers and reviews, no books devoted to immunoblotting of proteins from a practical point of view have been published until now. We have, therefore, found it expedient to try to collect the sum of our present knowledge about applications and techniques of immunoblotting in this monograph.

Volume I focuses on technical descriptions (Sections 1 to 7). Volume II focuses on experimental applications (Section 8), and Clinical applications (Section 9). Volume I is built up like the chronological progress of an immunoblotting experiment, starting with chapters about separation methods and ending with chapters on detection principles and artifacts. Much attention is given to detailed descriptions and recipes, so that the book can be of direct use for bench work in the laboratory. In the application chapters (Volume II), we have tried to select some representative topics. A chapter on future aspects closes the book.

The volumes deal with immunoblotting but not with DNA, RNA, or ligand blotting. There are two exceptions: a chapter on lectin blotting and a chapter on cell blotting, which have been included to exemplify the versatility of the technique. No further attempts to discuss the heterogenous group of ligand blotting have been made.

Immunoblotting is now an established technique and part of the standard technology of biological sciences, but it has been used in such a diversity of connections that no "authorized" version of the procedure exists. We have chosen to let specialists in different fields write about their own part of the spectrum. The inclusion of so many authors, all describing their personal variant of immunoblotting, we think especially increases the value of the book despite some unavoidable repetitions. This approach should give the researcher seeking a solution to a particular problem in immunoblotting a chance to find useful information. We also hope that the combination of techniques and applications in one handbook, besides illustrating the applicability of the immunoblotting technique, will be a source of inspiration for researchers working in other fields. Therefore, abbreviations have been avoided as much as possible to make the chapters appear intelligible and readable.

We have the sole responsibility for the edition of the chapters, as no editorial board has been involved. We are grateful to our fellow authors for their fine contributions and for granting us editorial license to obtain, we hope, uniformity in style and composition.

Cophenhagen, July 1986
Ole J. Bjerrum
Niels H. H. Heegaard

THE EDITORS

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Dr. Bjerrum was born 1944 in Copenhagen and obtained his medical degree from the University of Copenhagen in 1969. After finishing his medical internship at the University Hospital, he studied protein chemistry at the Institute of Biochemistry, University of Uppsala, Sweden. At the Protein Laboratory, University of Copenhagen, he was appointed in 1971 as assistant professor and in 1974 as associate professor. Dr. Bjerrum has been director of the institution for 4 years. The Ph.D. degree from University of Copenhagen was obtained in 1978. In 1980-81 Dr. Bjerrum spent a year as visiting professor at the Department of Biochemistry and Molecular Biology, Northwestern University, Illinois. In 1987 he moved to Novo Industry A/S.

Dr. Bjerrum is a member of The Danish Academy of Natural Sciences, Danish Biochemical Society (vice-president 1983-84 and president 1984-86), Scandinavian Electrophoresis Society (council member since 1986), and International Electrophoresis Society (council member since 1986). He is on the editorial boards for *Journal of Biochemical and Biophysical Methods* and for *Electrophoresis*.

Dr. Bjerrum has presented over 30 invited lectures at International and National Meetings, approximately 40 guest lectures at Universities and Institutes, and organized 10 international training courses and workshops. He has published more than 110 research papers and edited handbooks on immuno-biochemical methodology. His current major research interest is the structure and function of membrane proteins and immunoassay technology.

Niels H. H. Heegaard, M.D., was born 1959 in Copenhagen and obtained the medical degree from the University of Copenhagen in 1986.

He is now appointed to the University Hospital for his clinical internship. Simultaneously, he is doing research at the Protein Laboratory, University of Copenhagen, where he has been associated since 1981.

He has presented lectures at International Meetings, acted as a referee for International Journals, and participated in arrangement of and teaching of postgraduate courses in membrane protein chemistry. The focus of his current research is on methodology development, and on the influence of ageing processes and hematological disorders on red cell membrane proteins.

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Section 1

IMMUNOBLOTTING — GENERAL PRINCIPLES AND PROCEDURES

Niels H. H. Heegaard and Ole J. Bjerrum

INTRODUCTION

A specific technique and the term "blotting" were first joined in 1975 when E. M. Southern[1] described the transfer of electrophoretically separated single-stranded DNA from gels to an immobilized state on a membrane. The approach was soon applied to RNA by Alwine et al.[2] and in 1979 Renart et al.[3] and Towbin et al.[4] introduced the blotting of proteins by means of capillary and electrophoretic transfer, respectively. The concept of immobilization added a new dimension to analytical electrophoresis. Prior to this, direct biochemical characterization of individual proteins buried within the separation support had been difficult and unsatisfactory, particularly in tight gels, such as polyacrylamide.

Earlier, immunochemical characterization of gel-separated proteins had attracted special interest. An array of immunoelectrophoretic techniques was developed for the purpose of identification and characterization such as crossed immunoelectrophoresis, crossed sodium dodecyl sulfate polyacrylamide gel immunoelectrophoresis (SDS-PAGE IE), crossed immunoelectrofocusing, and immunofixation (procedures described extensively in References 5 to 7). A common characteristic is that they work in agarose. However, for immunochemical analysis of proteins separated by high resolution electrophoresis in polyacrylamide gels new tricks had to be invented (see the following text and Table 1). Thus, analysis by (SDS-PAGE) of an antigen in a mixture is possible after immunoprecipitation with a monospecific antibody. It does not necessarily require a precipitating antibody,[8] but coprecipitation and cross-reactions or aggregation of proteins make it difficult to identify unambiguously the unique antigen relating to the antibody preparation. Other methods employ analysis by crossed immunoelectrophoresis of excised bands from SDS-PAGE,[9] or the reverse where excised bands from crossed immunoelectrophoresis are investigated by SDS-PAGE.[10] Both need precipitating antibodies. Diffusion of antibodies into the gel by applying the antibodies directly onto the gel surface followed by detection with radioactively labeled anti-antibodies (primary binding assays, Burridge gel technique),[11] does not demand precipitating antibodies. The procedure is time consuming (days), needs relatively large amounts of protein (micrograms), and is hampered by the restrictions of antigen accessibility determined by the pore size of the gel matrix (i.e., the acrylamide concentration and crosslinking). Moreover, the long diffusion time reduces the resolution originally achieved by the gel electrophoresis. Better resolution was gained when SDS-PAGE was combined with electrophoresis or diffusion into an antibody-containing overlayed agarose gel (immunoreplica electrophoresis),[12] but these techniques also require precipitating antibodies and the resolution of the SDS-PAGE is not preserved during the different procedures.

The majority of the drawbacks mentioned above are eliminated when the proteins of the separation gel are transferred to a protein binding membrane (immobilizing matrix) followed by antibody incubation. In this way, the analytical potential of the separation techniques is expanded with the specificity and sensitivity of immunodetection. This idea was first practically employed for proteins by Towbin et al.[4] who introduced the technique of electroblotting from SDS-polyacrylamide gels to nitrocellulose sheets followed by immunodetection. An attempt to show the relationship between immunoblotting and other immunochemical techniques used for identification, characterization, and quantification of antigens and antibodies is shown in Figure 1. For a description of the present state of the technique, three recent reviews can also be consulted.[13,14]

Table 1
COMBINATIONS OF IMMUNOCHEMICAL DETECTION AND SDS-PAGE ANALYSIS BEFORE THE ERA OF IMMUNOBLOTTING

Modification	Ref.
Material from immunoprecipitation in solution analyzed by SDS-PAGE	8
SDS-PAGE followed by incubation with antibodies	11
SDS-PAGE followed by electrophoresis into a second dimension antibody-containing agarose gel	15
SDS-PAGE/2D-IEF-SDS-PAGE followed by diffusion into an overlayed agarose gel containing antibodies	12
Excised bands from SDS-PAGE analyzed by crossed immunoelectrophoresis	9
Excised immunoprecipitates from crossed immunoelectrophoresis subjected to SDS-PAGE and 2D-IEF-SDS-PAGE	10

Abbreviations: SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; 2D-IEF-SDS-PAGE; two-dimensional isoelectric focusing-SDS-PAGE.

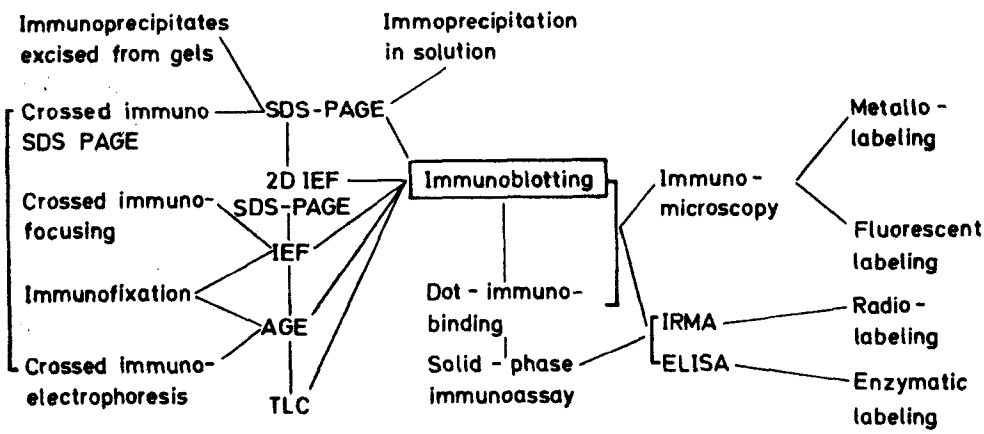

FIGURE 1. Relationship between immunoblotting and other techniques for immunochemical identification, quantification, and characterization of antigens. Abbreviations: AGE, Agarose gel electophoresis; ELISA, enzyme-linked immunosorbent assay; IEF, isoelectric focusing; IRMA, immunoradiometric assay; RIA, radioimmunoassay; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; TLC, thin layer chromatography; 2D, two-dimensional.

PRINCIPLES

A blotting experiment can, in principle, be delineated as a method where affinity interactions between ligands and acceptors take place on the spot and where molecular species have been rendered accessible and maintained in a pattern depending on their physicochemical properties. Biological material, e.g., proteins, can be immobilized on a membrane by wetting (dotting or spotting) or transferred by capillary, diffusional, or electrical forces. The material might be separated before the transfer. The binding forces of the membrane can be of a covalent and noncovalent nature. The main advantages of the blotting principle are due to:

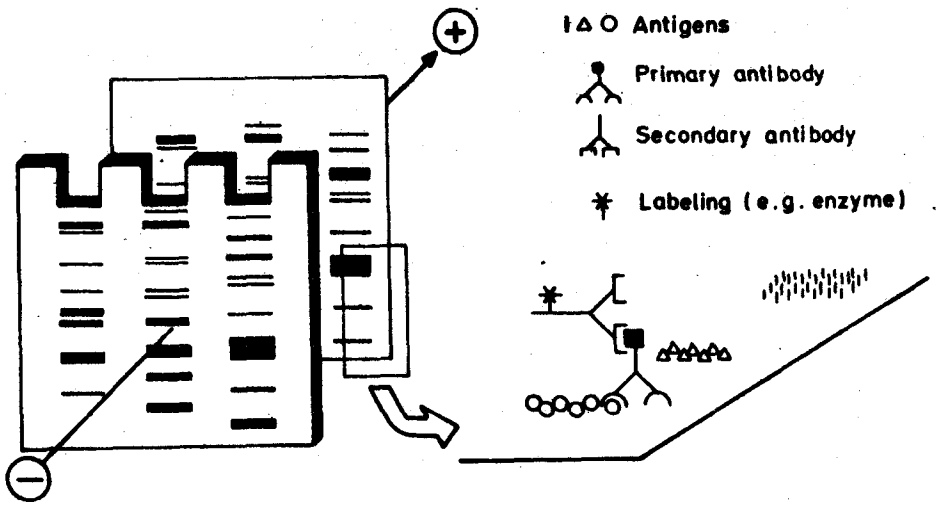

FIGURE 2. General principle of electroimmunoblotting.

1. Accessibility — The antigens are concentrated at the surface of the membrane texture.
2. Immobilization — Diffusion is avoided. The original resolution is thus preserved.
3. Time — Electroblotting can be performed in 1 hr (see Chapter 4.3.2).[16] The accessibility of the transferred proteins further shortens the time required for reactions with ligands. (In addition, the procedure normally does not involve especially difficult steps and most blotting membranes are easily handled.)
4. Economics — Small amounts of proteins are required (10 to 100 ng) as almost all of it is recovered on the blotting membrane ready for the ligand binding assays.
5. Flexibility — The blots might be stored and reused by probing with different ligands. The principle is applicable to all types of ligands (e.g., antigens/antibodies, receptors/hormones, glycoproteins/lectins) and electrophoresis principles (see Chapter 3) and other separation methods such as thin-layer chromatography.

The topic of these volumes is blotting of proteins and the detection by antibodies which is termed immunoblotting. Figure 2 shows the general principle of immunoblotting which can be modified in numerous ways.

TERMINOLOGY

We find that the term "immunoblotting" should be a common denominator for all blotting methods from all kinds of analytical supports with subsequent immunochemical recognition of the transferred species. Less used synonymous expressions would be immunoprinting, immunoreplicas, or immunotransfer. Hence, the words blot, replica, transfer, and imprint are also considered synonymous. When immunoblotting is connected with enzyme-based marker systems it might be called enzyme-linked immunotransfer blot (EITB).[18] The somewhat semigeographical terminology used in naming DNA-blotting Southern blotting after its inventor and followed by calling RNA-blotting Northern blotting and the electrotransfer of proteins Western blotting is not recommended. First, the names are confusing; second, they are not logical; and finally, the use of geographical terms is at the risk of inducing political controversies.[19] For precise descriptions it is preferable to define the blotting method by stating (1) the class of molecules transferred, (2) the method of transfer, and (3) the type

of immobilizing matrix. For example: protein transfer by electrophoresis to nitrocellulose, protein replica by vacuum-blotting to Zetaprobe, or protein blotting by diffusion to diazobenzyloxymethyl membranes. Direct application (spotting) of a protein onto a blotting membrane followed by probing with antibodies should be called dot immunobinding (see Chapter 2); [20] synonymous terms would be dot blot immunoassay,[21] spot immunodetection,[22] immunodot,[23] and antigen spot detection.[2] The term dotblotting, though it is very idiomatic, is not adequately descriptive since the transfer from a solution to a solid phase by pipetting is not considered a blotting method. Special modifications have their own names. Examples are filter affinity transfer (FAT), [25,26] a reverse blotting method, and lectin and cell blotting, where the detection steps are not based on immunological recognition (Chapters 8. 6, and 8.7, respectively).

EQUIPMENT

The basic equipment differs depending on the method of blotting employed. Consult the special chapters covering the different blotting methods. Some examples are shown in Figures 3, 4, and 5.

REAGENTS

Detailed descriptions of the sources of chemicals and other reagents are given in the individual methodology chapters in the form of laboratory-ready recipes. The appendix to this chapter contains a thorough description of buffers and chemicals used in our laboratory for immunoblotting of SDS-PAGE separated proteins onto nitrocellulose.

PROCEDURES

The main steps of the technique are briefly considered under the headings: transfer, membranes, and visualization.

Look for the technical descriptions and modifications of these steps in the relevant chapters. A detailed description of our routine procedure for electroimmunoblotting of proteins to nitrocellulose membranes is found in the Appendix.

Transfer

Fundamentally, all kinds of driving forces might be used to transport proteins from analytical supports to blotting membranes: diffusion, osmosis, electrical potential, centrifugal forces, vacuum pressure. The choice of transfer principle generally depends on the class of molecules involved, on the composition of the support nesting these molecules, on the available equipment, and on economic and time considerations. Almost all blotting experiments so far have been performed with capillary, diffusional, vacuum, or electrical forces, the latter being the transfer principle most used for blotting of proteins. The transfer principles are presented schematically in Table 2 and the build-ups of the apparatus are sketched in Figures 3A to E.

The optimal transfer step gives a quantitative elution in the shortest possible time with retention of biological and antigenic characteristics. This is achieved to varying degrees with the different techniques. Blotting by diffusion, capillary forces, or by vacuum suction have no restrictions concerning the choice of buffer, and if the blotting membrane itself has no special requirements the buffer chosen should provide the best conditions for binding, renaturation, and solubility of the proteins. However, elution is not easily controlled or reproduced with any of these methods, and resolution might be lost during the diffusion and capillary blotting processes as a consequence of the long elution times. The poor elution forces restrict the use of diffusion and capillary blotting methods to agarose gels.

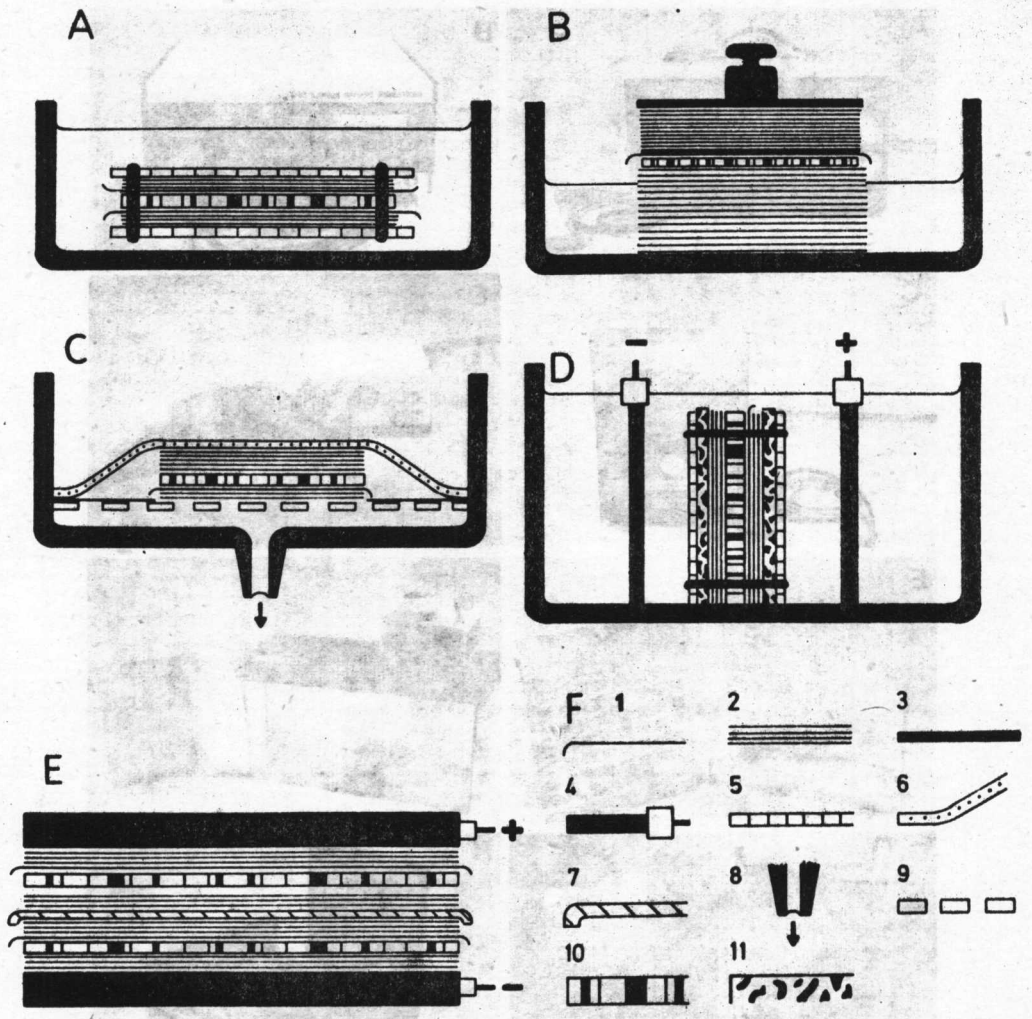

FIGURE 3. Transfer techniques in immunoblotting. A, diffusion; B, capillary flow-convection; C, vacuum-convection; D, electroblotting in buffer tank; E, semidry electroblotting; F, explanation of symbols (1, nitrocellulose sheet; 2, filter paper; 3, electrode; 4, electrode terminal; 5, plexiglass grid; 6, rubber sheet; 7, dialysis film; 8, outlet; 9, porous support; 10, gel; 11, sponge).

In electroelution, the direction and rate of transfer depend on the pH of the transfer buffer and placement of electrodes. The efficiency of elution depends on the composition of the separation medium (e.g., a gel), transfer time, size of the proteins, field strength, and on the presence of detergents. Application of special techniques like *in situ* digestion by pronase (see Chapter 4.3.4),[27] partial chemical breaking of the gel before blotting,[3] or widening of the gel pores by employing aqueous or urea-containing buffers for preequilibration of the gel also make elution more efficient. The binding of proteins to the blotting membranes depends on the characteristics of the proteins and membrane (see Membranes), presence of detergents, substances (like methanol in the case of nitrocellulose) which enhance the binding capacity of the membrane,[4] and on elements of the buffer interacting with the binding sites on the blotting matrix.

FIGURE 4. Equipment for electroblotting experiments. Power supplies (A to D) and transfer apparatus (E to H). (A) Battery charger 6 V/12 V, 5 A (Einhell). (B) Destainer power supply 12 V/24 V/36 V, equipped with timer (Pharmacia Fine Chemicals). (C) Power supply for electroblotting in buffer vessels 200 V, 0.6 A/60 V, 1.8 A (Bio-Rad). (D) Power supply for semidry electroblotting, variable voltage, 200 mA with 1 hr timer (Kem-En-Tec). (E) Simple electroblotting apparatus. Buffer vessel: glass jar, electrodes, and sandwich holders; stainless steel bathroom gratings (courtesy of Dr. Ib Rode Pedersen). (F) Gel destainer (Pharmacia Fine Chemicals). Plexiglass vessel with platinum anode and stainless steel cathode. (G) Electroblotting apparatus (Bio-Rad) with polycarbonate vessel, platinum electrodes, and locking gel cassette with hinged plexiglass sheets. (H) Semidry electroblotting apparatus (JKA-Biotech); graphite electrodes, plexiglass mounted.

At a given pH a more rapid and efficient transfer is obtained by use of higher field strengths for which reason the power supply should have a variable voltage and a timer. The upper limit depends on the heat dissipating abilities of the system in question and might be increased by using buffers of low ionic strength which generate less heat.[28] Large buffer volumes, cooling, and stirring can also reduce the temperature increase.