Techniques in Life Science and Biomedicine for the Non-Expert
Series Editor: Alexander E. Kalyuzhny

Biji T. Kurien

Western Blotting for the Non-Expert

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Techniques in Life Science and Biomedicine for the Non-Expert

Series editor

Alexander E. Kalyuzhny, Bio-Techne, Inc., Minneapolis, MN, USA

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Biji T. Kurien
Oklahoma Medical Research Foundation
Department of Veterans Affairs Medical Center
University of Oklahoma Health Sciences Center
Oklahoma City, OK, USA

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Preface

Series Editor Dr. John Walker's invitation in 2006 to edit a volume for the Methods in Molecular Biology series started the journey that has resulted in several works on western blotting. When we had just published a new volume in 2015, Series Editor Dr. Alexander E. Kalyuzhny sent an invitation to write/edit a volume on western blotting for the non-expert. Since Dr. Kalyuzhny had been a repeat contributor to our work, I could not pass up on this invitation. Torn between inviting authors a third time to contribute the chapters they had already submitted and editing the chapters already in possession, I finally decided to add some chapters personally and edit and simplify available chapters as much as possible for this volume. However, prior commitments did not permit the serious start of this work until the last part of 2020. Thus, I gratefully acknowledge that this work contains many of the methods developed by authors of the Methods in Molecular Biology series' volume on western blotting.

Simplistically, western blotting involves a series of simple steps which results in colored protein bands' appearance as though by magic by adding a substrate. As can be seen from this volume, western blotting can be a fun way for high schoolers and undergraduate students to create art on a new canvas, write secret messages, or even see their fingerprints appear as though by magic. On a more serious note, this method has remained a popular protein detection method since its inception in 1979. Western blotting and subsequent immunostaining have played a crucial role in advancing the proteomics field of immunobiology, neuroscience, to drug discovery. Now, the area has grown tremendously that the researcher has a variety of ways to carry out the protein blotting process. This volume begins with western blotting's exciting history and provides simple instructions to carry out the basic method and other protein blotting procedures.

Oklahoma City, OK, USA Biji T. Kurien, M.Sc., M.Phil., Ph.D.

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About the Author

Biji T. Kurien completed his M.Phil. and Ph.D. studies in 1989 at the University of Madras, India, under Professor R. Selvam's mentorship. After moving from India to the United States, he first joined Dr. Hiroyuki Matsumoto's laboratory in 1989. He then joined Dr. Robert H Broyles' laboratory in 1992, both in Oklahoma City. From 1993 to 2010, he worked as an associate research scientist/senior research scientist at the Oklahoma Medical Research Foundation, Oklahoma City, with Dr. Hal Scofield and from 2010 to 2017 as associate professor of research at the University of Oklahoma Health Sciences. Currently, he works as a professor of research. His research interests include the study of free radical-mediated damage in systemic lupus erythematosus and Sjögren's syndrome and the role of the nutraceutical curcumin in autoimmune diseases. His publication record includes numerous publications in national and international peer-reviewed journals. Besides, he edited several volumes such as *Protein Blotting and Detection* and *Protein Electrophoresis* as part of the Methods in Molecular Biology series.

Chapter 1
Western Blotting: How It Began

Scientists knew a lot about proteins, ribonucleic acid (RNA), and deoxyribonucleic acid (DNA) by the year 1975. Drs. Watson and Crick had already won the Nobel Prize for describing the molecular structure of DNA. The genetic code had been deciphered. Nobel laureate Dr. Frederick Sanger developed a method in the early 1950s to determine the amino acid units that made up a protein structure. Now, he was set to invent an elegant method to sequence DNA in 1977.

However, the macromolecules DNA, RNA, and proteins had not been visualized properly on a stable support, enabling further manipulation of these molecules. Of course, these macromolecules had been visualized in gel electrophoresis, but due to the gels' fragile nature, it was impossible to manipulate the macromolecules further.

1.1 Gel Electrophoresis

Gel electrophoresis was the precursor to procedures like Southern blotting, northern blotting, and western blotting.

Gel electrophoresis got a big boost with the discovery of starch gels for separating macromolecules and introduced with it the notion of molecular sieving. How this procedure came into being makes an interesting story. Professor Oliver Smithies was already experimenting with protein separation using a moist potato starch-grain bed. However, this procedure was not very effective. He stumbled upon the ability of starch to gel while assisting his mother with the laundry as a child. He observed that the hot liquid she made to starch the collars of his father's shirts set into a gel when left standing. This childhood observation made Dr. Smithies think that if he heated a starch solution to boiling and allowed it to cool, proteins could migrate through the resulting jelly that could be detected by staining with protein stains. The starch gel's utility was greatly influential in the development of polyacrylamide gel electrophoresis (PAGE). The polyacrylamide support medium was first introduced

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in 1959. Molecular weight and surface charge of biomolecules are two noteworthy factors causal to the separation in PAGE. This technique has been widely used to separate proteins and their characterization since the 1970s [1].

After experimenting with DNA electrophoresis using moving boundary techniques in 1964, scientists became enamored by methods that separated proteins in synthetic gel supports. Around the same period, investigators started to employ similar matrices to separate RNA and DNA molecules by molecular mass. Agar, agarose, polyacrylamide, and agarose-acrylamide combination gels were used for separation matrices [2]. Dr. Laemmli built on these successes by introducing the discontinuous buffer system for polyacrylamide gels and the Laemmli lysis buffer to prepare samples for SDS-PAGE [3]. Thus, he garners almost all the citations for protein gel electrophoresis currently.

1.2 Southern Blotting

The use of agarose gel electrophoresis formed the basis for the beginning of Southern blotting, a process used for transferring DNA fragments to a nitrocellulose membrane. In 1973, Sir Edwin Southern, a British scientist, set the ball rolling for beginning a revolution in transferring biomolecules to membranes.

When Dr. Southern was a child, he became the favorite of Mrs. Laycock, his first teacher at primary school. In those days, documents were copied in a primitive manner. The method was to write a master copy in thick violet-colored ink on a shiny, highly carded paper. This was then laid on a dish of "evil-smelling gel" so that the ink saturated into the gel and reproductions could be prepared by placing blank sheets on the gel to soak out the ink's imprint. Sir Southern became very acquainted with this technique since Mrs. Laycock engaged him to utilize it to make copies of examination papers. This experience enabled Southern to learn a great deal, and as he recollects the origin of Southern blotting, he wonders whether "there was an echo of this experience at the light bulb moment" in 1973.

In 1973, Dr. Southern was seeking a means of purifying genes from eukaryotic DNA. In that time period, cloning was not available, and only a few methods were available to purify genes. The availability of type II restriction endonucleases, described earlier by Tom Kelly and Ham Smith, greatly helped Dr. Southern. This enzyme had the capability of cutting the huge DNA molecule into smaller pieces. These enzymes provided a powerful way of fractionating DNA when combined with agarose gel electrophoresis. The hard part was finding specific genes in the complex smear of fragments from running the digested eukaryotic DNA on a gel. The difficult part was finding the bands of 5S genes in the complex smear of fragments that resulted from running the digest of eukaryotic DNA on a gel. Cutting the gel into slices, eluting the DNA, and analyzing the fractions by filter hybridization were tedious and gave very noisy backgrounds that hid the genes' low signal. Sir Southern and the group tried cutting the gel into thin slices, following by eluting the

DNA and analyzing the fractions by filter hybridization. However, they found that it was tedious and provided very noisy backgrounds that hid the genes' low signal.

Next, Sir Southern toyed with the idea of dissolving the gel with concentrated sodium perchlorate while it was laid against a filter. The idea was that the DNA would stick to the filter and thus retain the pattern of bands. Then, the filter-bound DNA would be amenable to the commonly used hybridization procedure. So Dr. Southern began the experiment using a strip of gel floating on a raft of cellulose nitrate membrane on a solution of sodium perchlorate. As Dr. Southern sat and observed, he failed to see any sign of the gel dissolving. Instead, he noticed a "bead of liquid forming on top of the gel." He quickly realized that the gel was permeable and that it would be possible to "soak out the DNA by a blotting process." So, he placed the membrane on top of the gel, overlaid it with filter papers, and used a buffer instead of sodium perchlorate. This experiment worked the first time, and Dr. Southern was thrilled to visualize sharp bands on the autoradiograph in the hybridization experiment.

Following electrophoretic separation of the DNA molecules on an agarose gel, Southern placed the gel on a membrane and allowed the DNA molecules to diffuse out of the gel and bind to the nitrocellulose membrane by passive diffusion. He was then able to use a radioactive probe, made out of synthesized DNA nucleotide polymer called a primer and a radioisotope, to detect the DNA molecules on the membrane. This procedure came to be known as Southern blotting, the process of transferring DNA molecules to a stable membrane [4].

The method became popular quickly, being in extensive use long before the *Journal of Molecular Biology* published it. Interestingly, however, his first attempt at publication resulted in rejection for the sole reason of it being a "methods paper." Therefore, he had to spend around one year to obtain more biologically related data before it was accepted.

1.3 Northern Blotting

After the introduction of Southern blotting, Nygaard and Hall found that nitrocellulose membranes did not retain ^{32}P-labeled RNA. Alwine and David Kemp derivatized cellulose powder sheets instead of using the derivatized cellulose powder developed by Barbara Noyes. They used this sheet on RNA separated by electrophoresis in agarose gels in the same way as Southern had done for DNA. The authors called the procedure northern blotting as a play of words concerning the Southern blotting procedure [5].

1.4 The Triumvirate of Western Blotting

In 1979, three scientists independently worked on developing a process to transfer proteins to membranes. Dr. Jaime Renart and colleagues developed a diffusion-mediated process to transfer proteins to a solid support [5]. Dr. Harry Towbin and Dr. Neal Burnette experimented with better ways to achieve this transfer.

Dr. Towbin and colleagues toyed with the idea of preparing a replica of a protein gel on a membrane, like Southern's DNA blotting. In the laboratory, he used an electrophoretic destainer to remove surplus stain from gels. The gels were positioned between two grids, and upon applying current at a right angle to the plane of the sheet, the charged dye molecules quickly came off the gel. Then, Dr. Towbin thought, "Well, the proteins are also charged – would they act like the dye?". He put together the basic things needed for the transfer – pipette tip holders, Scotch-Brite scouring pads, and rubber strings. These items were used to build a sandwich that is still being used for western blotting. Dr. Towbin carried out a series of simple experiments and demonstrated that the nitrocellulose membranes consistently captured the proteins as they left the gel. When he developed the first blot from a gel with antibodies with bands and saw the band that darkened within seconds, Dr. Towbin became so much excited that he felt like a child holding a sheet of paper over a flame and finding secret messages inscribed in invisible ink [6].

Around the same time, Dr. Neal Burnette placed gels with resolved protein bands in direct contact with derivatized and later with unmodified nitrocellulose sheets to see if the proteins would diffuse out of the gel onto the membrane. Initially, he had problems with the nonspecific binding of immunoglobulin and protein A reagents to the nitrocellulose, which he overcame by using a blocking agent (he used purified bovine serum albumin from which immunoglobulin had been removed). However, it became clear that passive transfer was slow, inefficient, and produced diffusion-mediated band-spreading of the proteins.

After this, Dr. Burnette also independently used a method similar to that used by Dr. Towbin. He also used an old electrophoretic gel destainer to drive the proteins out of the gel and capture them with a nitrocellulose membrane. However, by this time, Dr. Towbin's paper regarding the transfer of proteins from gel to the membrane using electric current came to be published.

Meanwhile, Dr. Burnette prepared a manuscript, and following a discussion with Bob Nowinski, the name "western blotting" came into being. At this juncture, the publication of Towbin et al. [6] appeared. Even though Dr. Burnette's basic technique resembled that of Dr. Towbin's, Dr. Burnette believed several aspects of his western blotting (e.g., unmodified nitrocellulose, radiolabeled protein A detection, 2D separations, etc.) had sufficient importance to warrant publication of his manuscript.

When Dr. Burnette submitted the manuscript to *Analytical Biochemistry*, the journal editors rejected it without any option for resubmission. The rejection apparently stemmed mainly from the reviewers' feeling of the ordinary nature of the

contribution and, especially, to the frivolous quaintness in the name "western blotting."

However, Dr. Burnette had already sent preprints of the rejected manuscript to colleagues, who had then given them to others, and so on until it looked (even in that pre-electronic era of written communications) like this unpublished manuscript had obtained broader circulation than numerous published articles. This fact became apparent only after Dr. Burnette moved to the Salk Institute at the end of 1979. He spent a significant part of each workday answering questions over the telephone regarding the method and supplying decipherable copies of the preprint. The original manuscript that he had sent to a few colleagues had gone through several photocopy replication cycles as it proceeded its way from lab to lab, finally becoming hard to read. About 6 months of carrying out this "private journal club," Dr. Burnette finally called the editor in chief of *Analytical Biochemistry*. The chief editor finally agreed that the general immunoblotting method and the name "western blotting" had become commonly accepted and that the original rejection of his manuscript was perhaps unfortunate. The journal immediately accepted the resubmitted paper and published it a few months afterward [7]. Dr. Burnette offers this solace to those scientists who feel the pain of rejection from journals that it is worth observing that his paper "entered a small pantheon of the most highly-cited scientific articles, all of which were initially rejected for publication" [7].

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Chapter 2
Basics of Western Blotting

2.1 Introduction

A single cell has thousands of proteins (http://www.bionumbers.hms.harvard.edu/.

In scientific research, one needs to study the characteristic of a particular protein. This can be particularly important in disease conditions.

How do we go about achieving this challenging task? The work of many brilliant scientists simplified this procedure.

The vexing problem of identifying specific proteins in a cell was solved by the introduction of the procedure known as protein blotting or western blotting. Cells obtained from tissues or cells grown outside the human body in a plastic flask are first busted to release its contents. The cell contents, which include a mixture of proteins, are first separated from each other according to the protein's size on a gel using a procedure called gel electrophoresis. The usefulness of the high separating power of sodium dodecyl sulfate-polyacrylamide gel electrophoresis was limited because the gel matrix-separated proteins were difficult to access with antibodies or other probes. Also, the gel is fragile and can be easily broken when handled. Therefore, there was a need to transfer the gel's proteins to more stable support that could be accessed easily with antibodies. This is where the procedure of western blotting became a powerful and important procedure for the study of proteins following electrophoresis, particularly proteins present in low amounts in the cell. The transfer produced a replica of the protein pattern in the gel on the membrane support. This allowed the analysis of specific proteins on the membrane using antibodies in a procedure called immunoblotting.

In brief, the transfer of proteins or nucleic acids to membranes is called "blotting." This term covers both "spotting" (deposition of purified protein sample manually on the membrane) and protein transfer from the gel proteins that are separated on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gels] [1, 2]. DNA transferring from agarose gels to the membrane support through

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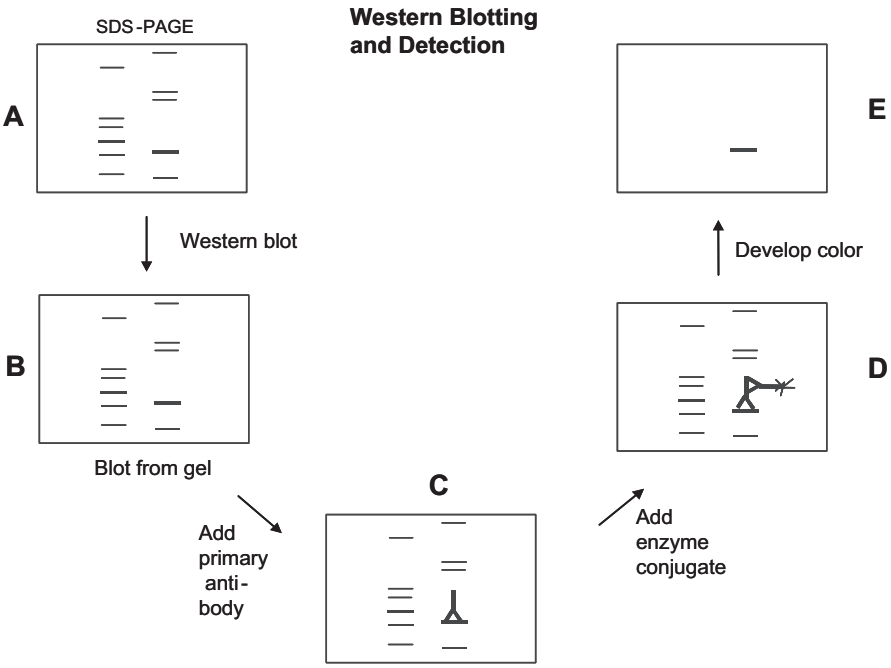

Fig. 2.1 Schematic representation of western blotting and detection procedure. (**a**) Unstained SDS-PAGE gel prior to western blot. The bands shown are hypothetical. (**b**) An exact replica of SDS-PAGE gel obtained as a blot following western transfer. (**c**) Primary antibody binding to a specific band on the blot. (**d**) Secondary antibody conjugated to an enzyme (alkaline phosphatase or horseradish peroxidase) binding to primary antibody. (**e**) Color development of a specific band. [Kurien, B. T., Scofield, R. H. (2015) *Methods Mol Biol.* **1312**, 17–30]

capillary action is known as Southern blotting. Protein blotting grew from DNA (Southern) blotting [3] and RNA (northern) blotting [4]. The term "western blotting" was invented to describe [5] this procedure to retain the "geographic" naming tradition started by Southern's paper [3]. The blotted proteins are an exact copy of the pattern found in the gel [6]. Western blotting has been useful as the starting step for several experiments. The use of antibody probes directed against the proteins bound to the membrane (immunoblotting) has transformed the arena of immunology (Fig. 2.1). Dot blotting is the term given when proteins are spotted directly on the membrane.

Western blotting [1, 2] has the following explicit benefits; (a) wet membranes are flexible and are cool to handle compared to gels, (b) there is an easy availability of the proteins attached on the membrane to different probes (antibodies, etc.), (c) only a small amount of reagents are required for studying transferred proteins, (d) multiple copies of a gel are possible, (e) it allows extended storage of transferred protein patterns, and (f) the same protein transfer can be used for numerous sequential analyses [7–9].

Since the time it was first described, protein blotting has developed a lot. Currently, scientists have several ways to transfer proteins to membranes [10]. However, it is essential to take care of:

1. The efficiency of blotting or transfer of proteins.
2. Retention of antigen on the membrane during processing.
3. The final detection/amplification system to achieve good sensitivity of western blot.

Flaws at any of these steps can compromise results [11].

2.1.1 Efficiency of Blotting

One needs to consider the type of gel to be used, the molecular weight of the proteins that are to be transferred, and the kind of membrane used to figure out the best way to obtain an efficient transfer of proteins from a gel to solid membrane support. Using the softest gel concerning acrylamide and cross-linker that provides the required resolution is the best option. The transfer will be more complete and quicker by using thinner gels. However, care must be taken to see that one does not use gels thinner than 0.4 mm in thickness. The use of ultrathin gels may cause handling problems [12]. It has to be noted that proteins with a high molecular mass transfer poorly following SDS-PAGE and can lead to low detection levels on immunoblots. However, scientists have used heat, special buffers, and partial proteolytic digestion of the proteins before transfer to improve the transfer of such proteins [11, 13–17].

2.2 Supports for Protein Transfer

A wide variety of solid supports is available for transferring proteins from gels. This includes excellent solid supports such as glass and plastic to latex and cellulose that are porous. The most commonly used supports for blotting are microporous surfaces and membranes like cellulose, nitrocellulose (NC), polyvinylidene difluoride, cellulose acetate, polyethane sulfone, and nylon. The microporous surfaces have some exceptional properties that make them suitable for "western blotting." These properties are (a) large volume to surface area ratio, (b) high protein binding capacity, (c) short- and long-term storage of immobilized molecules, (d) ease of processing by allowing antibodies to interact with the immobilized proteins, (e) lack of interference with the detection strategy, and (f) reproducibility. These properties also make them useful in the post-genomic era for high-throughput assays. A huge amount of data can be obtained by employing high technology things like robotics, sensitive detectors, control software, etc. [2, 4, 14, 18, 19].