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Western blot: a tool in the biomedical field

Western blot: una herramienta en el área biomédica

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Abstract

The Western blot technique, also called immunoblotting, is a high-sensitivity and semi-quantitative molecular technique that allows the analysis of a specific protein or protein profile. This is a technique for separating proteins according to their charge and molecular weight in polyacrylamide gels and their subsequent transference to a solid membrane, in which a specific antibody detects the protein. Four decades after its creation, the Western blot technique has gained an essential role in the diagnosis of various medical conditions, as well as an everyday tool in biomedical research.

Resumen

La inmunotransferencia de proteínas, o Western blot, es una técnica molecular semicuantitativa de alta sensibilidad que permite la inmunodetección específica de una proteína o perfil de proteínas. Esta técnica se basa en la separación de proteínas de acuerdo con su carga y peso molecular en geles de poliacrilamida y su posterior transferencia a una membrana sólida, en la que un anticuerpo específico detecta la proteína de interés. Cuatro décadas después de su creación, la técnica de Western blot ha ganado un papel importante en el diagnóstico de varias condiciones médicas, además de constituir una herramienta de cada día en la investigación biomédica.

Introduction

The Western blot (WB) analysis is an analytical technique used to detect proteins. The technique is based on the separation of proteins by molecular weight (MW) and electric charge using a gel electrophoresis. Afterwards, the proteins are transferred to a synthetic membrane and they are exposed to antibodies of interest (primary antibodies). Most of the time, the primary antibody must be coupled to a second antibody attached to a developer that identifies the protein of interest *(Figure 1A)*.[1-3] The WB emerged in 1970, when Ulrich Laemmli used the principle of molecule separation through an electrical gradient using polyacrylamide gels. At the end of this decade, Towbin (1979) described the transfer of proteins from polyacrylamide-urea gels to nitrocellulose sheets, being Burnette two years later, who employed the term Western blot (WB) for the technique using polyacrylamide gels and sodium dodecyl sulfate (SDS-PAGE).[4-7] Nowadays, the WB

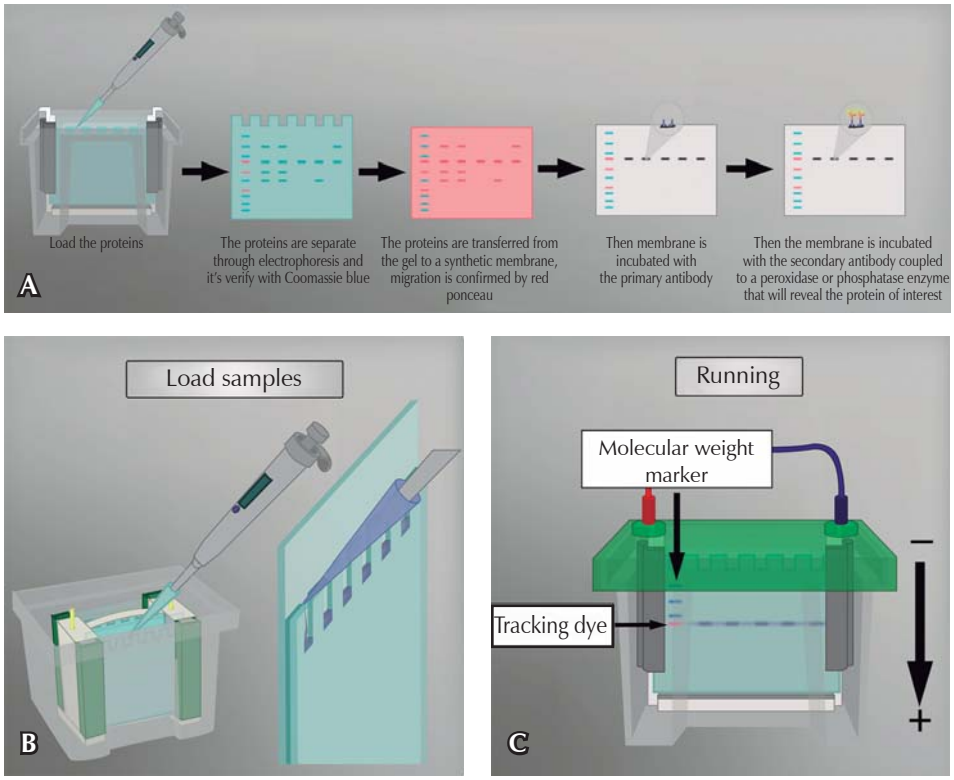

Figure 1.

(A) The steps of the western blot. a) Loading the protein into the gel, separation by electrophoresis, transfer and immunomarking. **(B)** The protein sample is loaded using a micropipette for precision (20-40 µg). **(C)** The separated proteins are visualized with a tracking dye (bromophenol blue) and molecular weight marker.

has become a key technique in the biomedical field for diagnosing infectious, autoimmune, rheumatic and oncologic diseases.[3,4,8] This review is centralized in describe and analyze the main characteristics of the WB and the importance of this technic in the biomedical field.

Cell lysis to extract protein

The WB is a versatile technique that detects proteins from animal and herbal tissues, cell cultures, bacteria and yeast. In order to obtain trustworthy results, a good protein extraction is required, which can be performed by mechanical and chemical methods. Within the mechanical methods, the most used are sonication, homogenization using abrasives, and disintegration with glass or metallic beads.[9] The chemical methods include the use of buffers capable of solubilizing the proteins, since they contain ionic detergents such as sodium dodecyl sulfate (SDS), deoxycholate and cetyl trimethylammonium bromide (CTAB). It is also possible to use other detergents, such as non-ionic or zwitterionic chemicals, and the choice depends on the speed of the lytic effect, as well as the extraction efficiency desired. For example, SDS can lyse cells in seconds, but it can denature the proteins. Triton X-100 is a non-ionic detergent that can lyse cells smoothly and slowly, avoiding protein denaturation and protein complex breakdown; this method is recommended for assays involving protein structure or protein activity. The zwitterionic detergents,-like 3 [(3-cholamidopropyl) dimethylammonium] -1-propanesufonate can be used without affecting the net charge or the charge of the solubilized protein. One of the most widely used buffers for its efficiency solubilizing proteins is radioimmunoprecipitation assay (RIPA), which contains 50 mM Tris-HCl pH 7.5, 150 mM NaCl, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, 5 mM EDTA, and a commercial inhibitor of proteases.[10] In HT-29, the colon cancer cell line, this reagent has showed a high efficiency solubilizing cytoplasmic proteins.[11] Besides RIPA, there are buffers for extracting cytoplasmic proteins, such as Tris-Triton (10 mM Tris pH 7.4, 100 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 10% glycerol, 0.1% SDS, 0.5% deoxycholate) and the 20 mM Tris-HCl pH 7.5 for cytoplasmic proteins. The purpose of using lysis-buffer solutions supplemented with proteases inhibitors is to maintain the protein structures. Finally, after the lysis process, the sub-cellular fractions are separated trough differential centrifugations, at the end; it is possible to obtain membrane proteins in the pellet and soluble proteins in the supernatant.[4,12]

Protein quantification

Before performing the electrophoresis, the protein content needs to be quantified in order to homogenize the amount deposited in the well of the gel and to determine the protein concentration. A large amount of protein can saturate the immunodetection and yield unspecific results.[13]

There are different methods to quantify proteins. The most used are Lowry, Bradford and bicinchoninic acid (BCA). These colorimetric assays are based on the color change when some protein amino acids react to different reagents.[13,14]

Lowry protein assay

This method is characterized by the use of Follin reagent and copper, it is detected in a wavelength of 750 nm, and the color intensity is based on the protein concentration.[15]

Bradford protein assay

This method uses Coomasie Brilliant Blue dye, which reacts in the presence of proteins, and the color change is detected in the wavelength range of 465-595 nm.[15]

Bicinchoninic acid assay

The protein assay with bicinchoninic acid (BCA) is highly sensitive and combines the reaction of proteins with Cu^{2+} ions in an alkaline method in the presence of a reagent that detects Cu^{+} ions with a high sensitivity, known as BCA. Its variation is minimum. It has been reported that the macromolecular structure of the protein and four specific peptides (cysteine, cysteine, tryptophan and tyrosine) are responsible for the change in color in the samples into a purple hue that can be detected at a wavelength of 562 nm.[14,15]

The electrophoresis gels

Several methods have been used to separate proteins, most notably those involving cellulose acetate paper, a starch gel that improved the resolution, but did not provide control over the pore size. More recently, polyacrylamide gels are used to control the pore size thanks to the regulation of acrylamide (referred to as % T) and bisacrylamide (referred as % C) percentage. With a higher percentage of acrylamide (10-20% T), the pore size is reduced and the gel is optimal for separating low molecular weight proteins (less than 50 kDa), whereas gels with a lower percentage of acrylamide (< 10% T) are recommended for separating higher molecular weight proteins (greater than 100 kDa).[16-18] Adding ammonium persulfate and tetramethylethylenediamine (TEMED) generates free radicals that accelerate the acrylamide polymerization. The versatility of the running buffers for polyacrylamide gels provides a fast method to separate proteins in order to identify them.[18]

Types of gels

Protein separation through polyacrylamide gels can be performed under naive or denaturing conditions. The non-denaturing polyacrylamide gels (ND-PAGE) keep the protein structure in a tridimensional shape and the separation is based on the electric charge, size and shape. In order to maintain the protein structure, a non-reductive, non-denaturant buffer solution is used, such as tri-glycine within a pH range between 8.3 and 9.5, or tris-borate at a pH of 7.0 to 8.5, and tris-acetate with a pH of 7.2 to 8.5.[19] In contrast, in the denaturing polyacrylamide gels (SDS-PAGE), the protein structures are dissociated in peptides using a combination of an anionic detergent (SDS), a reducing agent (beta-mercaptoethanol), and heat. SDS binds and provides negative charge to proteins and breaks the hydrophobic interactions, whereas beta-mercaptoethanol and the heat break the disulfide bridges (Cys-S-S-Cys) and thiol groups (Cys-SH) present in the polypeptide chains, ensuring protein dissociation *(Figure 2A)*.[4,16,18,20] Once the gel is polymerized, a suitable concentration of protein (20-40 μg) is deposited in the well created with the comb (10-15 well) *(Figure 1B)*. Protein supernatant volume and concentration can depend on the well capitation and thickness (0.75-1.5 mm).[21]

Discontinuous and continuous buffer systems

Depending on the type of buffers, there are two electrophoresis systems, the continuous buffer system of Weber and Osborn, in which the same running buffer is used in the single separator gel and the tank, and the discontinuous buffer or Laemmli system, which employs two types of gels: a «stacking gel» (large-pore) made with 4% of acrylamide and a «resolving gel» (small-pore) that polymerizes on the stacking gel. In the discontinuous system, the buffers used in each gel are made is different with different pH and ionic strength, and the running buffer employed is a different one. The most widely used is the discontinuous system,

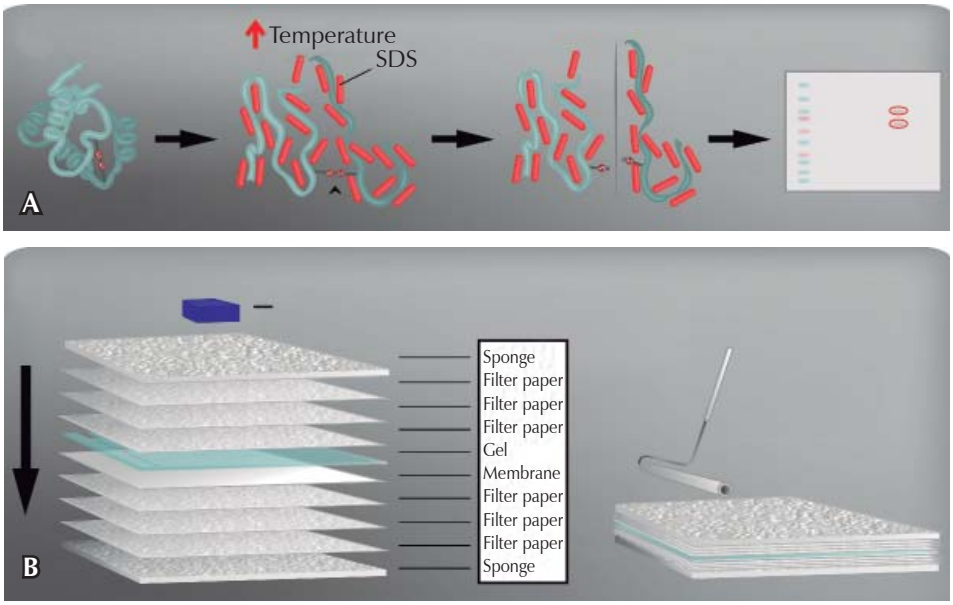

Figure 2.

Denaturation and transfer. **(A)** Protein cleavage requires an anionic detergent, SDS, to provide them with a negative charge, beta-mercaptoethanol, and high temperature to break the disulfide bonds of the polypeptide chains. **(B)** The transfer takes place from the negatively charged proteins in the gel to the membrane following the same distribution from the gel.

where protein mobility in the stacking gel is mediated by the chloride ions (Cl^-) in the gel and the glycine ion (Gly^-) in the sample buffer, resulting in a zone with low conductivity and voltage difference that favors the protein concentration before the transfer to the resolving gel, where the basic pH promotes glycine ionization for migration along the gel and favors protein separation based on the size.[22]

Sample preparation

Before carrying out the electrophoresis, the samples are heated in a temperature range between 70-95 °C in a buffer known as «loading buffer», which has the purpose of providing weight, density and color to the sample in order to allow for the sample to load into the well and avoid any leakages. This buffer contains 62.5 mM of Tris-HCl at a pH of 6.8, 2% SDS, 10% glycerol, 100 mM dithiothreitol (DTT) and 0.01% bromophenol blue. Among these components, DTT or beta-mercaptoethanol are the reducing agents that break the disulfide bonds. On the other hand, SDS allows the denaturation of proteins into a primary structure and covers them with a negative charge, making it possible to separate the proteins based on their molecular weight. Additionally, the reduction and denaturation allows for the antibody to reach its union site.[22]

In the gel, proteins with a lower molecular weight (MW) migrate to the bottom and those with a higher MW stand in the top of the gel. The protein migration throughout the gel can be observed thanks to the bromophenol blue dye besides the protein molecular weight marker. The last one helps mainly to identify the protein of interest because this marker contains purified proteins with different MW *(Figure 1C)*.[4,16,17,23,24]

Transfer of proteins to the membrane

In this technique, proteins must be transferred to a resistant solid support, like a membrane, so they can easily be handled and used in the immunodetection process with specific antibodies.[25]

The transfer principle is similar to the polyacrylamide gel electrophoresis, i.e., negatively charged proteins migrate towards the positive electrode under the influence of an electric field.

The membrane is interposed between the gel and the positive electrode *(Figure 2B)*, and the proteins are retained in the membrane with the same distribution they had in the gel. The transfer can be performed through one of two systems: semi-dry or wet. Both systems are characterized by the close contact between the gel and the membrane, but the wet process requires a higher volume of transfer buffer and longer exposure in lower temperature (2-12 h at 4 °C) compared to the semi-dry system (7-30 min at room temperature).[22,25]

In both types of transference, the use of different buffers is recommended, such as Tris-glycine in a range of several concentrations at a pH of 8.3-9.2 and SDS (0.025-0.1%), with or without 20% methanol, bicarbonate buffer with at a pH of 9.9, SDS (0.025-

0.1%) and 1x TBE (Tris-borate 90 mM, EDTA 1 mM). The Tris-glycine buffer, pH 8.3, containing SDS at 0.1% with methanol at 20% is the most widely used buffer, especially because it is the universal buffer without methanol for the SDS-PAGE gel. The methanol has two main roles: triggering the dissociation of SDS to the protein and drastically improving the adsorption of proteins in the membranes in the presence of SDS. However, these effects may vary among proteins.[26]

The supports that are frequently used in the transfer are microporous surfaces, such as nitrocellulose membranes or polyvinylidene difluoride (PVDF), which provide different features that affect the result.[16,25] These membranes should have important properties, such as a high binding capacity, ease of storage of immobilized molecules in the short and long term, and reproducibility.[22,26] The PVDF membrane is stronger and provides better binding to the protein, unlike nitrocellulose. Nevertheless, the nitrocellulose membrane provides less background noise. In comparison to the nitrocellulose membranes, the PVDF membranes need to be moistened in methanol or ethanol before using them due to their highly hydrophobic nature, and they do not have any surfactant. Recently, in order to reduce the background noise, membranes of low autofluorescence are used.[3,17]

Immunodetection

Once the proteins are retained in the solid phase (membrane), the non-specific and empty sites in the membrane are blocked. The most widely used blocking agents include those that contain proteins because they can bind to the empty sites on the membrane. This reduces the background noise because the antibody added can only bind to the sites of the proteins in question. Some protein-containing blocking agents include horse or fetal calf serum and Tween-20 (a detergent) if there is so much background. However, non-ionic detergents should be used carefully, since they can solubilize the proteins transferred to the membrane. Nonetheless, non-fat milk and bovine serum albumin (BSA) are the most widely used. Non-fat milk has a sub-optimal behavior in the analysis of phosphorylated proteins because it contains casein, a phosphoprotein that may compete for the antibody target, thus reducing the intensity of a non-specific signal.[11]

The previously blocked membranes are incubated with the primary specific antibody of the protein of interest. The antibodies used in WB can be polyclonal antibodies that recognize several regions of the antigen or monoclonal antibodies that recognize a single region of the antigen, as shown in *figure 3*.[16]

Most of the time, the primary antibody is not coupled to any developing agent. Therefore, incubation is required afterwards with a secondary antibody. The secondary antibodies bind to the primary antibodies that are not bound to antigens, and they are selected depending on the species in which the antibody is made primary.[17]

Immunodetection of proteins

Chemiluminescent immunodetection

Immunodection by chemiluminescence uses peroxidase or phosphatase enzymes coupled to a secondary antibody that radiates energy in the form of light generated by a reaction of luminescent compounds (luminol). This allows for the detection of the antigen-antibody binding *(Figures 4A and 4B)*. The light reaction is developed in autoradiographic film, and more recently, it is digitally captured using a digital camera to identify the protein of interest.[16]

The sensitivity of the chemiluminescence immunodetection technique depends on the linearity of the detection, which generally is present in loads

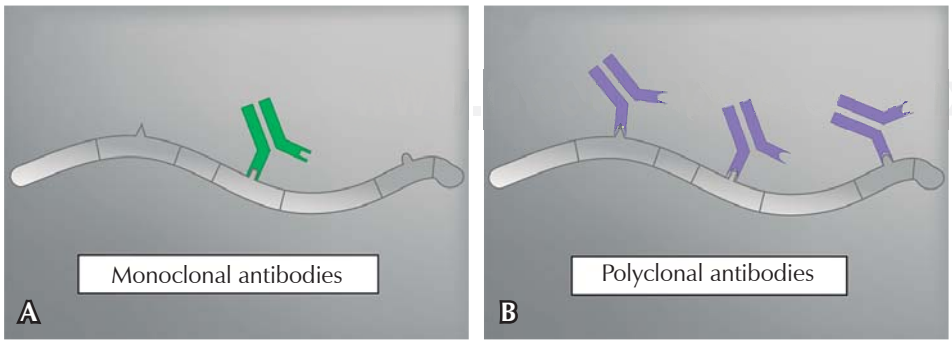

Figure 3.

Recognition difference of primary antibody. **(A)** Monoclonal antibodies recognize a region of the antigen. **(B)** Polyclonal antibodies recognize several antigenic regions.

lower than 5 μg.[27,28] The signal saturation is directly related to the protein expression, considering this immunodetection method as «semi-quantitative», since the saturation point in the signal is a critical factor for determining the differences in the expression levels of the samples and could lead to inaccurate measurements.[28] The captured image allows the pixels quantification of each band that were obtained through a densitometric analysis, using the analysis software included in digital systems *(Figures 4C and 4D)*.[7,16,17,29]

Immunodetection with fluorescence

Recently, a fluorescent immunodetection method has been proposed, in which secondary antibodies couple to a fluorescent marker or fluorophore and emit light when they are excited, creating a lineal-detection signal *(Figures 5A and 5B)*. A digital camera with filters of different wavelengths detects the emitted light. Similar to the images revealed by the chemiluminescence technique, the images captured are analyzed with the same principle. However, the result is quantitative because of the signal stability, in contrast to the chemiluminescence immunodetection method *(Figures 5C to 5E)*.[1,28,29]

Western blot applications in the biomedical field

In the clinical field, the WB technique serves as a diagnostic method for various infectious, autoimmune, and oncological diseases.[16,17,30] This technique is one of the most important tools used in medical research. In the infectious diseases area, protein detection through WB supports the diagnosis of various diseases, like

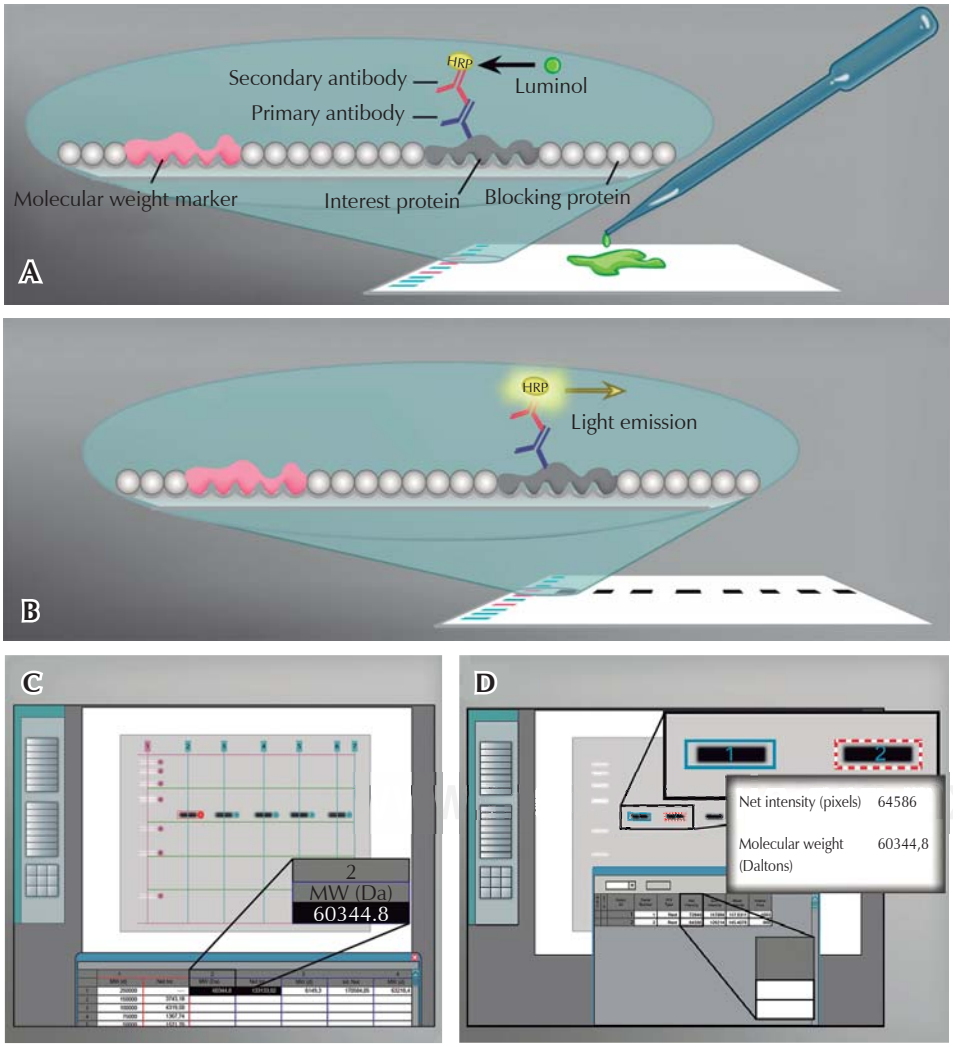

Figure 4.

Luminescent Immunodetection. **(A)** Immunodetection carried out with a luminescent compound (luminol). **(B)** The reaction between peroxidase or phosphatase and luminol emits light, allowing for the antigen-antibody detection. **(C)** The densitometric analysis determines the molecular weight and **(D)** the number of pixels on the band of interest.

visceral leishmaniasis, where the strain of the parasite causing the disease can be identified using the WB.[31] In the diagnosis of congenital toxoplasmosis, WB has allowed for the identification of proteins involved in the immune response against the toxoplasma parasite.[32,33] The WB has been used as a diagnostic test for Lyme disease (caused by the *bacterium Borrelia burgdorferi*) transmitted to humans through the bite of an infected

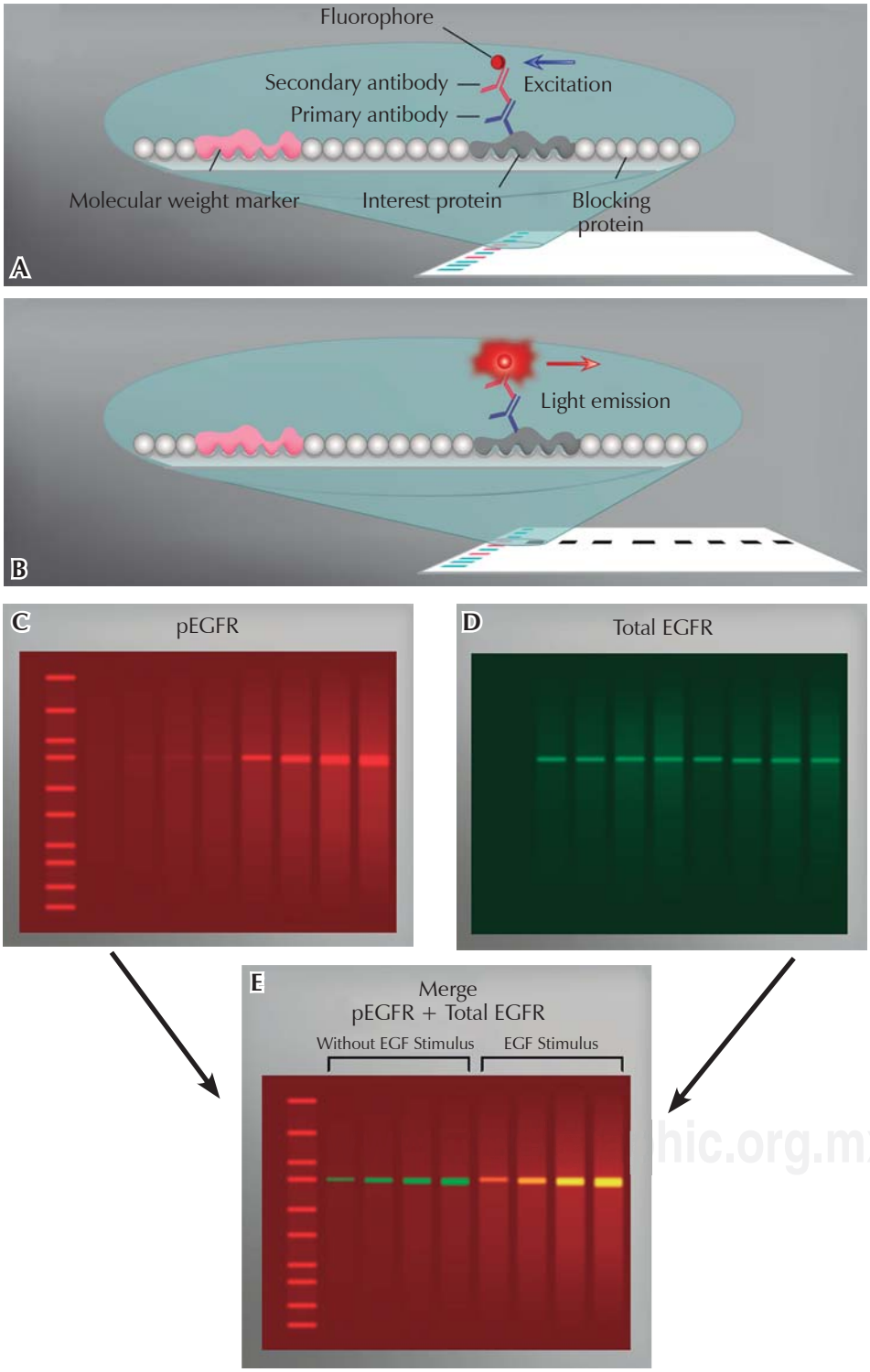

Figure 5.

Fluorescent immunodetection. **(A)** Fluorescent immunodetection performed with secondary antibodies coupled to a fluorophore. **(B)** When excited, the fluorophore emits light for detecting the antigen-antibody interaction. **(C)** The immunofluorescence images allow for the individual analysis of a protein in its phosphorylated form, as well as **(D)** the full analysis **(E)** together (merge).

blacklegged tick, and it is used to identify a protein array characteristic of the bacteria in serum.[34-37]

Moreover, the WB analysis of serum samples of patients with melioidosis also called Whitmore's disease (caused *Burkholderia pseudomallei*) has been able to characterize the *bacterium* proteins causing the disease.[38]

The WB has been used to confirm the diagnosis of pulmonary tuberculosis, identifying the mycobacterial antigens characteristic of *M. tuberculosis*.[39] Additionally, the WB has been considered a high-sensitivity diagnostic test for detecting congenital syphilis in samples of asymptomatic children.[40] The WB has served as a 100% reliable test to diagnose the herpes virus infection, detecting 22 serum samples with the virus out of a total of 27.[41] The WB has proven to be a non-invasive and highly sensitive diagnostic test detecting 47.8% of patients with duodenal ulcer caused by *Helicobacter pylori*.[42] In the hydatid disease caused by the cestode *Echinococcus granulosus*, the WB has been able to detect specific antibodies for proteins of 39 and 42 kDa in 96% of patients with hydatid disease.[43] Recently, this technique has been established as a pre and postsurgical diagnostic test for detecting hydatid cysts because of its demonstrated high sensitivity of 100%.[44]

The WB has been used to identify proteins in synovial fluid and serum, allowing for the diagnosis of clinical symptoms of rheumatoid arthritis and osteoarthritis.[45-47]

Wang et al. (2011) used the WB to determine the expression levels of the follistatin-like protein 1 (FSTL1) as a possible biomarker of damage in the articular level in patients with knee osteoarthritis, since it is involved in the prevention of mechanisms of articular degradation *in vivo* and *in vitro* models. Their findings revealed a relationship between the overexpression of FSTL 1 and the osteoarthritis degree.[48]

Recently, α1-antitrypsin (A1AT), a protease inhibitor, has been reported to be a great load control for the WB in samples of synovial fluid of patients with osteoarthritis, since it is abundant and constant.[49]

This technique has managed to show the relationship between the protein expression levels of the leukocyte antigen system class II (HSP60, HSP70, p53 and collagen II) and the development of rheumatoid arthritis (RA) in patients sera.[50]

The WB technique has been a key factor in the identification of the citrullinated protein (CCP) as a marker for RA in samples of synovial fluid, given its high sensitivity (80%) and specificity (98%) values.[51]

In 2002, Salma et al. used the WB technique to detect proteins of the parasite *Sarcocystis* in sera samples of RA patients as positive markers for detecting rheumatic diseases.[52]

Recently, in samples of patients with laryngeal cancer, the WB showed for the first time overexpression of a protein called PFN1, responsible for metastasis of this carcinoma (laryngeal), considering it a biomarker for diagnosis and a therapeutic target.[53]

The WB help in the early diagnosis of liver cancer, identifying the 14-3-3 proteins expressed during progression to hepatocellular carcinoma in patients who have not been diagnosed with the disease.[54]

In esophageal cancer, the WB has allowed for detecting overexpression of the protein glutathione S-transferase omega 1 (GSTO1), which induces an immune response, considering this protein as a biomarker for early diagnosis of the disease.[55]

In a mouse model with Alzheimer's disease (AD), the WB technique identified the overexpression of a drug transporter called ABCG2, which allows the passage of a protein that causes a vascular damage called cerebral amyloid angiopathy (CAA), considering ABCG2 a biomarker for the CAA pathology in patients with Alzheimer's.[56] It has also played a key role in identifying seven prognostic biomarkers for prostate cancer that determine the aggressiveness degree.[57]

In muscular dystrophies, the WB has been recognized as a reliable technique for routine diagnosis.[58]

Besides these applications, the WB technique is consider as a helpful tool in several fields in the biomedical area, contributing in the boarding of different diseases in order to have a diagnostic and understand some physiopathological characteristics.[59]

Conclusions

For more than four decades, the WB technique has been used as a suitable tool in the biomedical research field due to its application in a wide range of different samples, using photodocumentation equipment that goes from the most simple approach of luminescence detection to the more sophisticated ones that are able to detect specific emission sources for specific fluorophores, which increases the cost, but yields more efficient results, making it a crucial tool for diagnosing and detecting several diseases, and for finding therapeutic targets, giving this technique a major importance in the clinic area.

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