Review

# Western Blot: A Valuable Immunoassay

Emilia Manole, Ionela D. Popescu,
Carolina Constantin, Simona Mihai, Gisela F. Gaina,
Elena Codrici, Alexandra E. Bastian and
Monica T. Neagu

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**Abstract**

Although an old analytical and quantitative technique, Western blot (WB) is still topical in detecting protein biomarkers from different biological samples, in the field of research as in pathology. In addition to the initial technology, new equipment and new WB techniques have been developed or updated over time to allow for shorter working times and better reproducibility or reduce troubleshooting. In this chapter, we present the situations in which the WB is the best choice for protein biomarker immunoassay but also the challenge it encounters in front of multiplexing microarray technology such as protein microarray, surface plasmon resonance imaging, Luminex multiplex assays and even the traditional ELISA. We also show technical innovations and optimizations that made WB faster and easier. However, immunoblotting is not only in a competitive relationship with other immunoassay techniques but also in cooperation with them, for a better biomarker identification in different pathologies, as we show.

**Keywords:** Western blot, protein biomarkers, SPRi, protein microarray, Luminex, ELISA

## 1. Introduction

In basic and medical research, increasing demand to analyze existing proteomic biomarkers and to find new ones, both for diagnosis and for personalized treatment, has led to the development of increasingly complex methodologies. Proteomic biomarkers are particularly useful for providing information on cellular signaling pathways, bringing early disease data and

monitoring treatment response or adverse effects. They can be monitored from body fluids such as blood, urine, saliva, cerebrospinal fluid and tissues (biopsy).

The necessity to analyze very small amounts of proteins present in biological samples, as well as the increase in the number of proteins requiring simultaneous, reliable, reproducible and significant investigations, led to the modernization of the existing techniques and to the appearance of some new methods of biomarker investigation and analysis. Western blotting is one of the old methods that changed, is adapted, is modernized over time but remained "on barricades" for protein biomarker investigation.

Immunoassay methodologies are the most commonly used tools in protein research, using the properties of antibodies to bind different protein domains and to mark them. There are some high sensitivity methods for validating proteomic biomarkers such as protein microarray, surface plasmon resonance, Luminex multiplex assays, ELISA and, of course, Western blot (WB). In recent years, many multiplexed immunodetection techniques have been developed to simultaneously investigate multiple proteins (from several tens to several hundreds), in the same sample and which are in very low amounts.

The Western blot (WB), also known as immunoblot, it is an analytical and quantitative technique for identifying specific proteins in many biological samples, liquid or tissue/cellular homogenates.

The WB technique brings concrete and useful information that cannot be offered by other immunoassay methods. If the target protein, present in the sample, is altered qualitatively or quantitatively, the band thickness is changed compared to a control being downregulated or overexpressed. More of this, the target protein band may be positioned above or below the control protein band, heaving a modified chemical structure: it is glycosylated, methylated, etc. or it is degraded. It is possible to have two bands instead of one meaning that the protein forms multimers. These WB results can also guide us to a genetic investigation in the case of partial deletion or duplication in the protein gene. In addition, the WB method allows a quick comparison of target protein expression in many patients in medical diagnosis.

In many cases, this technique is used in conjunction with other immunoassay methods, such as immunohistochemistry, ELISA or microarrays. In these cases, WB may represent a confirmation of the other methods' outcome or a thoroughgoing study of a smaller number of interest proteins, significantly modified, for research or diagnosis purposes (e.g. in HIV or prion disease).

More of this, in research, the validation of a protein modification is done by two or three different immunoassay techniques, which include the use of, for example, Western blotting, protein microarray and Luminex or ELISA. So these techniques not only can be completed, but they can validate their mutual results.

## 2. History of the Western blot technique

The WB technique was invented by Harry Towbin and coworkers Theophil Staehelin and Julian Gordon of the Friedrich Miescher Institute in Basel, Switzerland, in 1979. They used the method

to identify bacterial or chicken ribosomal proteins separated on polyacrylamide gels containing urea. They called this method "electrophoretic blotting technique" [1]. The WB name was given 2 years later by Neal Burnette, of the Fred Hutchinson Cancer Research Center in Seattle, Washington, which also brought some improvements to this method, including the use of SDS-PAGE gels. The name "Western" was inspired by the earlier name of other blotting methods, "Southern", named after the name of British biologist Edwin Southern, who published in 1975 a method for detecting specific DNA sequences [2] and "Northern" whose name was inspired by the name of the first blotting technique, "Southern", an RNA detection technique, developed in 1977 by James Alwine, David Kemp and George Stark at Stanford University [3, 4]. At that time there were no ready-made antibodies, so they made their first antibodies by injecting ribozyme proteins into animals like mice or goats. To achieve the nitrocellulose gel-membrane sandwich, Scotch-Brite scouring pads were used and a disposable micropipette tray as a structural support, all fixed with rubber bands. The transfer was done in an electrophoretic destaining chamber [1]. The situation was easier with the secondary antibodies because they could be bought at least for colorimetric or fluorescence detection. To reproduce the image on X-ray film, they had to improvise again, labeling their own prepared antibody with iodine-125 via the "chloramine T method" and exposing the labeled blots to film for 6 days.

Over time, the method has improved and has become easier to achieve, with nearly all materials commercially available: transfer devices, antibodies, precasting gels, digital imaging devices and so on. However, in the most part, as methodology, the technique proposed by the Towbin team remains valid after 38 years. Burnette, Stark and Towbin said after many years that they were surprised by the success and longevity of the method [5].

## 3. Evolution of the Western blot method over time

The WB technique is described in many booklets, articles or book chapters, and it is not our intention to make in this chapter another presentation of this methodology.

In summary, the Western blot method is a way to identify a target protein from a biological sample, a mixture of proteins, running it on polyacrylamide gel. The proteins in the sample are separated by SDS-PAGE gel electrophoresis, depending on their molecular weight. Because the gel is hard to handle, being fragile, the proteins are transferred to a membrane, usually nitrocellulose or polyvinylidene fluoride (PVDF), that maintains the gel pattern as a copy, which Towbin called "a faithful replica" [1]. The electrical current causes the transfer. For visualization of the protein of interest, the membrane is probed by a specific primary antibody, it binds the specific epitope of the protein, and it is labeled by addition of a secondary antibody recognizing the primary antibody conjugated with a detection reagent (fluorophore, enzyme, radioisotope). The visualization is done colorimetric, by chemiluminescence, on X-ray film, or directly in the membrane with the aid of an imaging system (**Figure 1**).

In order to be able to reuse a WB membrane that has already been exposed to primary and secondary antibodies, it is necessary to wash it. This operation is called stripping. Only membranes that have been treated with enhanced chemiluminescence (ECL) for protein visualization by chemiluminescence can be reused and not the membranes that have been treated colorimetric.

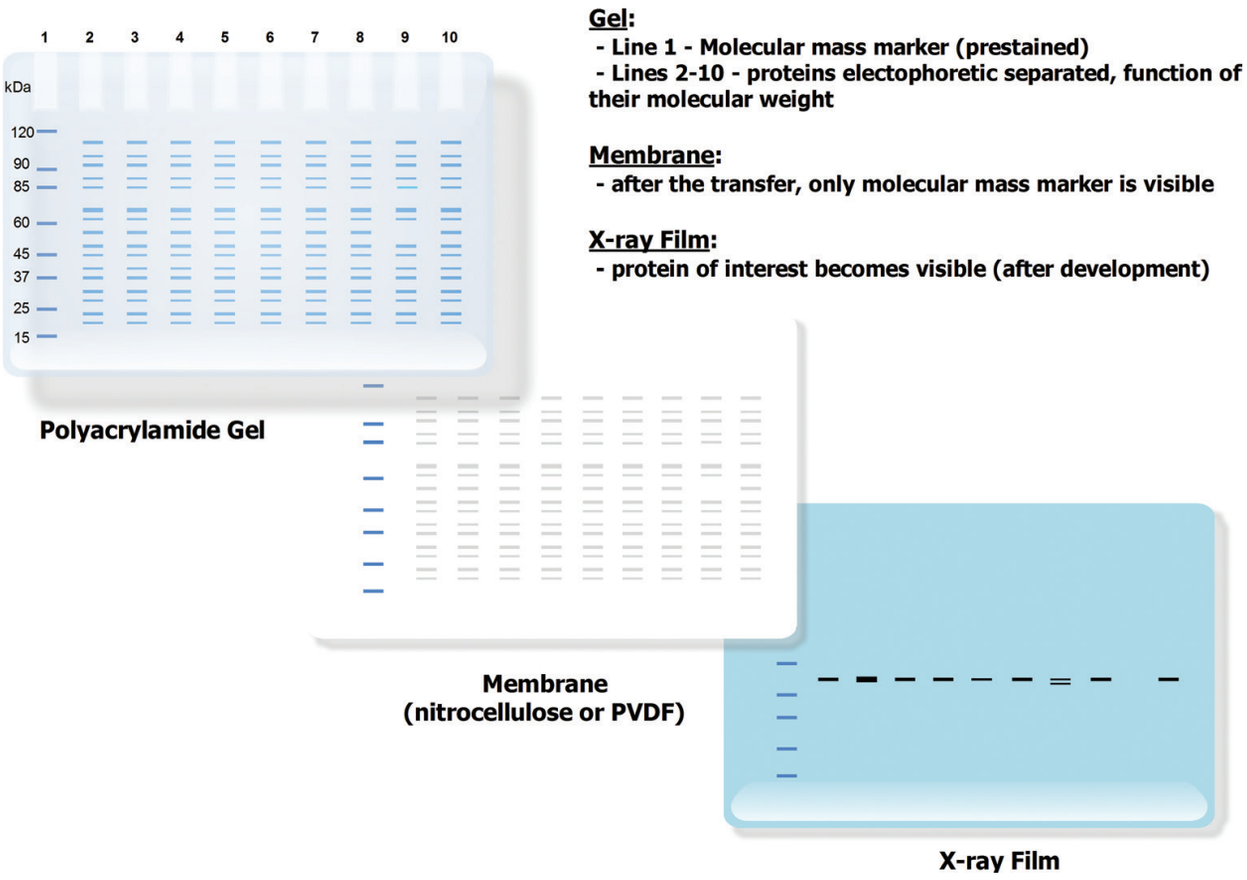


**Figure 1.** The main steps of classic Western blot method. (1) SDS-PAGE (polyacrylamide gel) electrophoresis—the proteins are separated depending on their molecular weight. (2) Transfer of the proteins on a membrane (nitrocellulose or PVDF). (3) Visualization of the protein of interest—the membrane is incubated with a specific primary antibody, then a secondary antibody recognizing the primary antibody conjugated with a detection reagent (fluorophore, enzyme, radioizotope). The visualization is done by chemiluminescence, on X-ray film.

This method is useful when we want to investigate more proteins on the same blot, for example, a protein of interest and a loading control protein. It saves biological samples, time and substances. Practically, instead of initiating a new WB, starting with other samples, a new gel, new electrophoresis and new transfer, after stripping, it follows another antibody incubation for another protein on the same membrane. The stripping procedure removes a small amount of protein. So it is advisable to first visualize a protein of interest that is in a small quantity in the sample and then, after stripping, a protein that is found in large amount in the sample, as is the loading control. For stripping, special buffers are used that can efficiently remove antibodies but do not remove too much amount of the proteins on the membrane.

To make the WB method easier to use, faster and more reliable over time, both users and manufacturing companies, responding to demand, have changed it in each of the abovementioned stages, from supplies to equipment. Here we present some examples:

### 3.1. Supplies

- Gels: precast SDS-PAGE gels ready to use; gradient gels for Western blot multiplex; stain-free gels with a rapid detection of proteins with stain-free enabled imagers; gels with a

special composition for small or heavy proteins (more or less SDS addition); native-PAGE gels that allow the separation of proteins without denaturing them, depending on several parameters such as charge, size and 3D structure, in the presence of a buffer that maintains secondary structure of proteins as well as native charge density; nonacrylamide, nontoxic, high-resolution electrophoresis precast hydrogel, etc.

- Membranes: nitrocellulose and PVDF, with different pore diameters (0.2–0.45 μm).
- Antibodies: there is a very wide range of commercially available primary and secondary antibodies with a high specificity for almost any type of experiment or diagnostic destination, fluorescent labeled antibodies, etc.
- Chemiluminescence kits with a higher sensitivity.

### 3.2. Equipment

Without intending to advertise any company, we will outline some of the improvements made by the different manufacturers, to highlight the vivid interest in Western blotting and the widespread applicability of this technique nowadays.

Each step of the WB was adapted to make it easier and faster. In the recent years, some equipment has been invented that has virtually changed all WB steps and are entirely automated. But let us take them one at a time.

The WB system size may vary, with electrophoresis/transfer tank, gels and membrane: mini, midi and large, depending of the investigated protein size and the time needed for separation (see **Table 1**). However, the vast majority of investigators use now the mini system, sometimes the midi one, because of the existence of gradient gels and more sophisticated devices (see below).

Transfer systems were developed by few companies to allow proteins the migration from gel to the membrane in different ways, using varying amounts of buffers: wet, semidry or dry systems (see **Table 1**).

To shorten the working time, a new semidry system was developed: Trans-Blot Turbo from Bio-Rad [6] reducing the transfer time at just 3 min instead of 1.5–3 h or more.

For a faster antibody incubation step, and an increased antibody-antigen binding, a Merk Millipore equipment has been invented. It reduces the whole time for this stage to 30 min (from at least 2 h at room temperature or overnight at 4°C for primary antibody and at least 1 h at room temperature for the secondary antibody, with the classic method), using a vacuum to actively drive reagents through the membrane: SNAP i.d.® 2.0 system [7, 8].

A series of membrane readers, imaging systems for chemiluminescent or fluorescent detection, have also been developed by a large number of manufacturers, to replace X-ray films. New digital technologies offer a good and rapid band visualization, avoiding underexposure or overexposure, as in the case of X-ray film developing. The images can be stored in a computer database. More of this, the imaging systems produce a linear signal over a wider dynamic range that can be analyzed with software that measure the optical density of the bands, ImageJ, for example.

| Technology | Blotting methods | Electrophoresis/transfer system—size | Number of proteins detected |
|---|---|---|---|
| Traditional/classic WB with traditional equipment. Many operations manually | Wet blotting | Mini—for mini gels of approx. 8 × 7 cm | Traditional—one protein identification |
| Traditional with the newest equipment—automatized | Semidry blotting | Midi—for midi gels of approx. 14 × 9 cm | Multiplex—more proteins in the same sample at the same time |
| WB in capillaries | Dry blotting | Large—for gels up to 20 × 20 cm | Far-WB—protein-protein interactions |

**Table 1.** Western blotting: types of WB technologies, types of blotting methods, size of WB systems and number of detected proteins.

All of this equipment are very useful and have many advantages, and it would be very good to have some of them in the laboratory, but our view is that the classic WB method still needs to be learned; once the equipment is broken or there are no funds for the consumables specifically related to some equipment, the classic method can save your work.

## 4. Other methods based on Western blotting

### 4.1. Multiplex Western blot

Western blotting had first developed to detect a single specific protein target in a complex mixture using alkaline phosphatase-conjugated antibody probes combined with colorimetric detection [1]. In the last few years, it has become a necessity to analyze multiple target proteins at the same time, in order to compare the expression of proteins involved in a specific pathology.

The first multiplex WB blot (MWB) experiment was optimized by Anderson and Davison [9] to study different muscle proteins involved in muscular dystrophies. This method allowed a simultaneous screening of multiple proteins with different sizes on a pair of blots, using a cocktail of monoclonal antibodies which permitted the identification of primary deficient and secondary deficient proteins in several muscle pathologies, knowing that the primary reduction of a protein causes the secondary reduction of another protein. The use of a MWB allowed establishing a biomarker profile for each patient, providing valuable information for diagnosis as well as for phenotype-genotype correlations.

The MWB method proposed by Anderson used a biphasic polyacrylamide gel (with different concentrations) system electrophoresis which separated the proteins with different molecular weights: molecular mass more than 200 kDa (e.g. dystrophin and dysferlin) in the upper part of the gel, with 5.5–4% polyacrylamide gradient, while proteins with molecular mass under 150 kDa (e.g. calpain-3, all of the sarcoglycans, caveolin-3) are separated in the lower phase, 7% polyacrylamide gel. Thus, for the first time, a gradient gel, with different concentrations, was developed for the study of several proteins with different molecular weights in the same sample.

*The applications of multiplex Western blot.* Introduction of this technique has revolutionized the medical diagnosis and opened new perspectives in biomedical research. Today, MWB is used especially in muscular pathology which requires simultaneous protein analysis.

*Advantages.* Simultaneous analysis of several proteins involved in different pathologies by MWB reduced cost and time for analysis. By this method proteins in the same sample could be determined and compared, as well as a secondary reduction of other proteins in a specific disease.

*Disadvantages.* The use of antibody cocktail requires a right dilution and right specificity of the primary antibodies. In order to avoid overlapping of the bands, a good choice of investigated proteins is required. Fortunately, the gradient gels are commercially available.

### 4.2. Capillary electrophoresis and capillary Western blotting

By this method the molecules are separated by size inside a capillary filled with an electrolyte. The advantage of the method is that the separating sieve matrix can be automatically pumped in and out because it contains rather unknown polymers than the typical cross-linked polymers for the gels. The big difference between the classic method and this one is that many samples can be run over and over again in an automated manner that saves a lot of time [5].

Capillary electrophoresis (CE) for the first time needs a smaller amount of sample than SDS-PAGE and offers a better resolution of protein size. Proteins travel down the capillaries and are spaced according to size. When the individual proteins reach the end of the capillary, they drop on a blotting membrane that moves along the capillary opening. It has been shown that classical protein standards such as carbonic anhydrase and lysozyme can be separated within an hour using only a few nanolitres of sample [10].

O'Neill et al. [11] have been able to capture the resolved proteins on the capillary wall by photochemically activated molecules. This method allowed immune complexes to be formed after electrophoresis, in the capillary. Chemiluminescence reagents flowed through the capillary, and the image was taken with a CCD camera.

In 2012, ProteinSimple launched Simple Western technology in a tool named Simon, commercially available. The protein mix was taken up by 12 capillaries filled with a sieving matrix, and the proteins were separated by size and immobilized on the capillary walls by exposing the capillaries to a source of ultraviolet light that activated proprietary chemistry. The separation matrix was removed, and the reagents for a standard immunoassay flowed into the capillaries. The primary antibody entered first, followed by the horse radish peroxidase-conjugated secondary antibody, which generated a chemiluminescent signal. Including sample preparation time, the entire automated process lasted 3–5 h. The instrument also allowed the quantitative measurements of the protein. Obtained data was more reproducible than classic WB. Among the latest developments of this equipment, we want to mention a new one that uses extremely small amounts of biological samples, 3 μl; the proteins analyzed in the same sample can have molecular weights from 2 to 440 kDa; it can analyze up to 25 samples in 3 h; and the data has a qualitative and quantitative certainty. A last generation tool from the same company, called Single-cell Western, can detect cell subpopulations that are not visible with a traditional WB. It can measure intracellular proteins, transcription factors, etc. A special chip

is used, on which a cell suspension is placed; the cells are captured in some partitions on the chip; and detections of proteins are done in these partitions [12].

### 4.3. Microfluidic Western blotting

This technology reduces much more the amount of the sample required for WB and also the length of the capillaries from centimetres to microns, using microfluidic channels. He and Herr [13] developed an automated immunoblotting method using a single streamlined microfluidic assay. A glass microfluidic chip, which has integrated a PAGE electrophoresis with subsequent in situ immunoblotting, allowed a rapid protein separation, directed electrophoretic transfer of resolved proteins to an inline blotting membrane, and a high-efficiency identification of proteins of interest using antibody-functionalized membranes [13, 14]. The system requires only 0.01–0.5 μg of protein.

The technology was improved with new performances: a short duration, all steps completed in 10–60 min, multiplexed analyte detection in a single sample, mass sensitivity at the femtogram level, high sensitivity for a starting sample concentration of 50 pM and a quantitation capability over a 3.6-log dynamic range, small device, a standard microscope slide form and reagent economy [15].

So far, the system is not commercially available, although improvements have been made over time. The microfluidic system was developed by Herr et al. to maximize blotted analyte signal, reducing the time for WB, and, finally, to determine the abundance of very low-level proteins in rare cells [16–18].

A team of Beijing researchers has succeeded in immunoblotting using a 10-channel microfluidic system that allowed the use of 10 primary antibodies to probe the membrane. The membrane is subsequently incubated with the secondary antibody. Thus, it is possible to analyze 10 different proteins instead of one for the same sample on the same membrane [19].

### 4.4. Dot blot

It is a method very similar to WB, but the proteins are not separated by gel electrophoresis. The samples are applied in small dots directly on the membrane and then spotted through circular templates. After membrane drying, the antibodies are applied. The visualization of target protein is made like at WB, chemiluminescent or colorimetric. Dot blot is used to test the specificity of some antibodies, to test the antibody concentration to be used for WB or to evaluate the presence of a target protein in sample before WB.

### 4.5. Far-Western blotting

It is used to detect a protein-protein interaction in vitro. Instead of the primary antibody for detecting the protein of interest, this method uses a non-antibody protein that binds to the protein of interest. Far-Western blotting detects proteins on the basis of the presence or the absence of binding sites for the protein probe. This method is important in characterization of protein interactions in biological processes such as signal transductions [20], receptor-ligand interactions or screen libraries for interacting proteins. Using this protocol, it is possible to study the

effect of posttranslational modifications on protein-protein interactions, use synthetic peptides as probes and examine interaction sequences and identify protein-protein interactions in any conditions, without using antigen-specific antibodies. The visualization of target protein is made by radiolabeling the non-antibody protein or by a specific antibody against this protein.

## 5. Western blot applications in clinical field

Among the first applications of the WB technique are testing the multidrug resistance mechanism in immortalized cells [21, 22], AIDS tests [23–25] and identifying Alzheimer's disease in brain extracts from patients [26].

From then to our time, many scientific papers were based on the WB technique. So we can make a jump to our days and see how much WB method is used in the last year. If we search on Google Academic with the keywords "WB in medical diagnostics" "from 2017", the search engine gives us 17,400 results. It is a relevant outcome to say that the old Western blot method is still very appreciated, not mentioning the research concerning the protein biomarkers.

To highlight the importance of WB technique in clinical diagnosis, we give some eloquent examples below.

Today, in medical field, WB serves as a diagnostic tool [27] for some infectious, oncological and autoimmune diseases and neurodegenerative disorders, like Alzheimer's disease [28] and muscular dystrophies [29].

Some of the infectious disorders identified using WB are visceral leishmaniasis [30], congenital toxoplasmosis [31], Lyme disease [32], pulmonary tuberculosis [33], Herpes virus infection [34], duodenal ulcer caused by *H. pylori* [35] and hydatid disease [36, 37].

For early diagnosis in cancer, WB helped in identification of laryngeal [38], liver [39] and oesophageal [40] cancers.

In autoimmune diseases, WB was used in rheumatoid arthritis [41, 42] and osteoarthritis [43].

For the viral infections, a WB method with a technical modification named Recombinant Immunoblotting Assay (RIBA) is used. The conventional protocol is changed by spotting recombinant antigen onto membrane strips which are used to screen samples from the patients for antibodies against virus. The separation of proteins by electrophoresis and the transfer onto a membrane are eliminated. These strips are commercially available ready to use in clinical laboratories [44].

WB was used some years ago for human immunodeficiency virus (HIV) and hepatitis B and C virus (HBV and HCV) infections, but the assay was producing false-positive results, so the diagnosis in these cases migrated toward nucleic acid testing [45].

The microfluidic WB platform developed by Herr's group revolutionized this system, being able to do thousands of independent WB on single cells in 4 h, as the Single-Cell Western platform. The advantages have been described above. These WB technologies could be adopted in the future by clinical laboratories.

## 6. Other immunoassay methods and their connection with Western blot

There are many modern immunoassay techniques as surface plasmon resonance imaging, Luminex xMap array, ELISA or protein microarray. They are used all to identify an analyte within a sample in different ways. Could there be a connection between these methods and the Western blot? Let us see.

### 6.1. Surface plasmon resonance imaging, lab-on-a-chip

Since our goal is not describing surface plasmon resonance imaging (SPRi) methodology, but comparing with WB and deciphering a possible interrelation between SPRi and WB, we will not insist very much on the description of the technique. But in order to analyze what we have proposed, we will make a brief description of the principle on which SPRi is based.

The first SPR immunoassay was proposed by the team Liedberg, Nylander and Lundström, in 1983 [46]. The SPR immunoassay method is label-free (unlike ELISA)—no label molecule is required for analyte detection [47]. Moreover, the measurement is done in real-time, which allows monitoring of the individual steps of this technology. SPRi is currently one of the most sensible platforms for studying a wide variety of interaction affinities [48, 49], involving nucleic acid sequences [50–52], peptides [53], proteins [54, 55] and carbohydrates [56]. It is possible to monitor hundreds of molecular interactions simultaneously.

The composition of a biochip consists essentially of a glass prism, coated with a thin gold film and a pre-functionalized surface chemistry. The sample to be analyzed is injected over the biochip, and the detection of a specific molecule can be performed by immobilizing a binding partner on the biochip. SPRi makes a non-labeling and a real-time detection of biomarkers [57].

The SPRi platform allows the quantitative detection of multiple simultaneous multiplex interactions, and many studies are based on this application for screening a variety of analytes in different array types.

In comparison to Western blot, SPRi has the advantage of investigating a large number of different analytes from the same sample (several hundred different spots can be placed on a biochip); after washing the biochip, it is possible to immediately analyze another sample. SPRi takes less time than WB. The disadvantage of SPRi would be that only liquid biological samples (blood, urine, cerebrospinal fluid, cell culture medium) can be analyzed; it does not analyze biological samples from different tissues/tissue lysates. Both techniques are analytical and quantitative. SPRi is very effective when there are many samples and many different interactions to analyze, but for a small number of samples or to demonstrate only one type of interaction between two proteins, for example, WB is more efficient.

### 6.2. Luminex xMAP array: Western blot cooperation in a multiplex era

An important improvement in the biological assay field was made in the late 1990s when Luminex xMAP technology was launched. xMAP technology combines the principles of ELISA and flow cytometry but goes beyond the limitations of solid-phase reaction kinetics

and is suitable for high-throughput, multiplex and simultaneous detection of different biomarkers within a very small volume sample. Bringing together advanced fluidics, optics and digital-signal processing with proprietary microspheres, xMAP technology became one of the fastest growing multiplex technologies. Featuring a flexible open-architecture design, xMAP technology enables configuration of various assays, quickly, cost-effectively and accurately, useful in clinical and research laboratories [58].

A key component of Luminex xMAP technology is represented by proprietary color-code polystyrene microspheres (beads) internally dyed with precise concentrations of two or three spectrally distinct fluorochromes. Through precise concentrations of these specific dyes, up to 500 distinctly bead sets can be developed, with a different spectral signature.

Based on fluorescent reporter signals, high-speed digital-signal processors identify each individual microsphere and quantify the result of every bioassay. The capability of adding multiple conjugated beads to each sample results in the ability to obtain multiple results from each sample [59].

There are different types of advanced detection platforms (as depicted in **Table 2**); therefore, various biomarker panels could be analyzed. Accordingly, validation of novel biomarkers into multiplex immunoassay panels confers an attractive prospect of simultaneous measurement of multiple analytes in a single patient sample, enabling progression monitoring and outcome prediction, even detecting major diseases in its earliest stages [60].

Some of our studies illustrate significant dysregulation in circulating levels of cytokines and angiogenic factors in brain tumors, with over threefold upregulation of IL-6, IL-1 beta,

| Technique | Advantages | Disadvantages | Use |
|---|---|---|---|
| Western blot | High specificity<br>High sensitivity<br>Quantitative<br>Automation potential | One or a small number of proteins/analytes at a time<br>Labor intensive/time consuming for the classical method. Resolved by the new equipments<br>Qualitative, quantitative, especially with the newest equipments<br>Difficult to transfer large or hydrophobic proteins—false-negative results<br>Difficult to automate—for the classical method. Resolved by the new equipments | To identify the presence of a small number of proteins in the same sample (Multiplex WB) or the presence of protein-protein interactions (Far-WB)<br>Confirmation of other screening method/antibodies validation |
| ELISA | Quantitative<br>High sensitivity<br>Medium specificity<br>Higher throughput than Western blot<br>Automation potential | One protein/analyte at a time<br>False-positive results<br>Labor intensive/time consuming/ high reagent use<br>Costly setup for automation | To quantify a single protein<br>Confirmation of other screening method/validation |

| Technique | Advantages | Disadvantages | Use |
|---|---|---|---|
| Bead-based array (e.g. Luminex® technology) | High sensitivity<br>High throughput and speed<br>Multiplex and customizable panels of analytes<br>Open-architecture design<br>Low time, labor and reagent use over traditional methods<br>Versatility<br>Flexibility | High cost for a specialized equipment and a validated antibody pair | To quantify (quantitative) multiple proteins/panels of analytes, in the same well from a small amount of sample<br>Clinical implementation—available IVD kits |
| Chip-based array:<br>Protein microarray | High sensitivity<br>Medium specificity<br>Highest throughput<br>Low time and reagent use | High cost for a specialized equipment and a validated antibody pair | To screen for changes across a large number of proteins, in the same well from a small amount of sample |
| SPRi | Very high sensitivity<br>Label-free<br>Real-time method<br>High throughput and speed<br>Multiplex and customizable panels of analytes<br>Open-architecture design<br>Low time, labor and reagent use over traditional methods<br>Low time and reagent use<br>Biochips reusable | High cost for a specialized equipment<br>Suitable for liquid biological probes | To screen and quantify (quantitative) multiple proteins interactions (and not only)/panels of analytes, from the same sample<br>Clinical implementation |

**Table 2.** Advantages and disadvantages of immunoassay methods presented in this chapter: Western blot, ELISA, Bead-based array, Chip-based array-protein microarray, SPRi.

TNF-alpha and IL-10 and up to twofold upregulation of VEGF, FGF-2, IL-8, IL-2 and GM-CSF, with implications in tumor progression and aggressiveness, and also involved in disease associated pain [61, 62].

Currently Western blot is used to validate/confirm the identified biomarkers and association between xMAP technology, and Western blot was remarked in many studies. Interestingly, one of them emphasized the improvement in diagnostic sensitivity of HIV infection in early stages using xMAP technology, increasing the chances of an early accurate diagnosis. Thus, a superior sensitivity of Luminex xMAP compared with Western blot was observed. Out of 87 confirmed HIV-positive cases, Western blot confirmed 74.7% sensitivity, while Luminex

xMAP identified 82.8% sensitivity ($p < 0.05$) [63]. Further advancements will be needed for a successful validation of current discoveries, and sustained efforts are necessary to expand the translation into clinical applications toward personalized medicine [64].

### 6.3. Western blot and ELISA, competition or completion?

Old traditional enzyme-linked immunosorbent assay (ELISA) was developed in 1971 by Engvall, Perlmann, Schuurs and Weemen [65, 66] and continues to be widely used as routine diagnostic method allowing quantitation of a large variety of proteins [67].

The single-plex ELISA, the most utilized assay method performed in 96- or 384-well plates, has played a prominent role in the quantitative and qualitative identification of analytes. There are direct and indirect ELISA detection methods with their own advantages and disadvantages. Direct ELISA could accurately quantify a specific molecule with high sensitivity from a wide variety of samples. Indirect ELISA detection is also more versatile in that primary antibodies can often be produced in one species and the identical labeled secondary antibody can potentially be utilized for subsequent detection.

The use of ELISA for assessing multiple analytes is time-consuming due to the large number of workflows occurring simultaneously and because of that new multiplexing technologies have been developed. Moreover, ELISA is designed as a solid-state immunoassay, and the use of a planar matrix can restrict immunoassay capacity, sensitivity and detection quality [68].

Conventional single-target assays ELISA and Western blot are suitable for biomarker validation but could be expensive, time-consuming and sample limiting. While most of the disease conditions may arise when only one single molecule is altered, more often it is the consequence of interaction between several molecules within the inflammation milieu; therefore studying the diseases necessitates a comprehensive perspective.

Moreover, with only one single molecule being evaluated, the researcher must know the target of interest and their importance in the studied disease state. If their model does not rely on a single target for detection or treatment, the techniques required for detection of these biomarkers are further limited by using a single-target method. Thus, high-throughput multiplex techniques need to be included, particularly in the early phases of drug discovery to assess molecule interactions, leading to the identification and development of novel biomarker panels with high efficiency, low costs and less sample [69].

### 6.4. Protein microarray and Western blot: an extension in microwestern array?

Protein microarray analysis is increasingly useful both for research purposes and for various biomedical applications, *including the niches ones* like evaluating markers of apoptosis activated by various therapies such as photodynamic therapy (PDT), assessment of epigenetic milieu or transcriptional activity in treated cells [70]. Thus protein microarray is a proteomic tool that can deliver high-throughput data for revealing new therapeutic targets [71].

Protein microarray history has spanned the last two decades, the basic principle being identical with ELISA, but there are several advantages such as spotting in terms of miniaturization, multiplexing and large data obtained in an ELISA equivalent time. Briefly, biological samples

of interest (e.g. serum, plasma, etc.) are incubated on a slide containing immobilized antibodies, proteins or peptides. An antigen-antibody reaction occurs between an analyte from the tested sample and the corresponding antibody from slide followed by the detection step through various methods (e.g. fluorescence-based detection). The slide is further scanned, followed by image acquisition, data processing and analysis. There are several classifications of this technique, but it could fall in two main categories: direct phase (e.g. antibody, protein, peptide array) and reverse or indirect phase where sample of interest is spotted on a slide and the corresponding antibody is further added. The general principle of any protein microarray is presented in **Figure 2**.

Among all these variants, the antibody array type is preferred in tumor research domain or in biomarker discovery/quantification due to technique's high versatility and reproducibility [72]. The reverse phase array format could also be used for biomarker discovery because of its specificity but has the disadvantage of being more laborious.

It is worth emphasizing that protein microarray could be customized in terms of number and multiplicity of tested analytes one achieving new research and clinical benefits through this technology. Thus, although fundamental research purposes prevail when it comes to array platforms, there is also a recent increasing trend in clinical research, diagnostics and even industry applications such as pharmacy or food. For instance, recent attempts are made in using array platform for autoimmune disease insights. Thus, novel antigen arrays have been

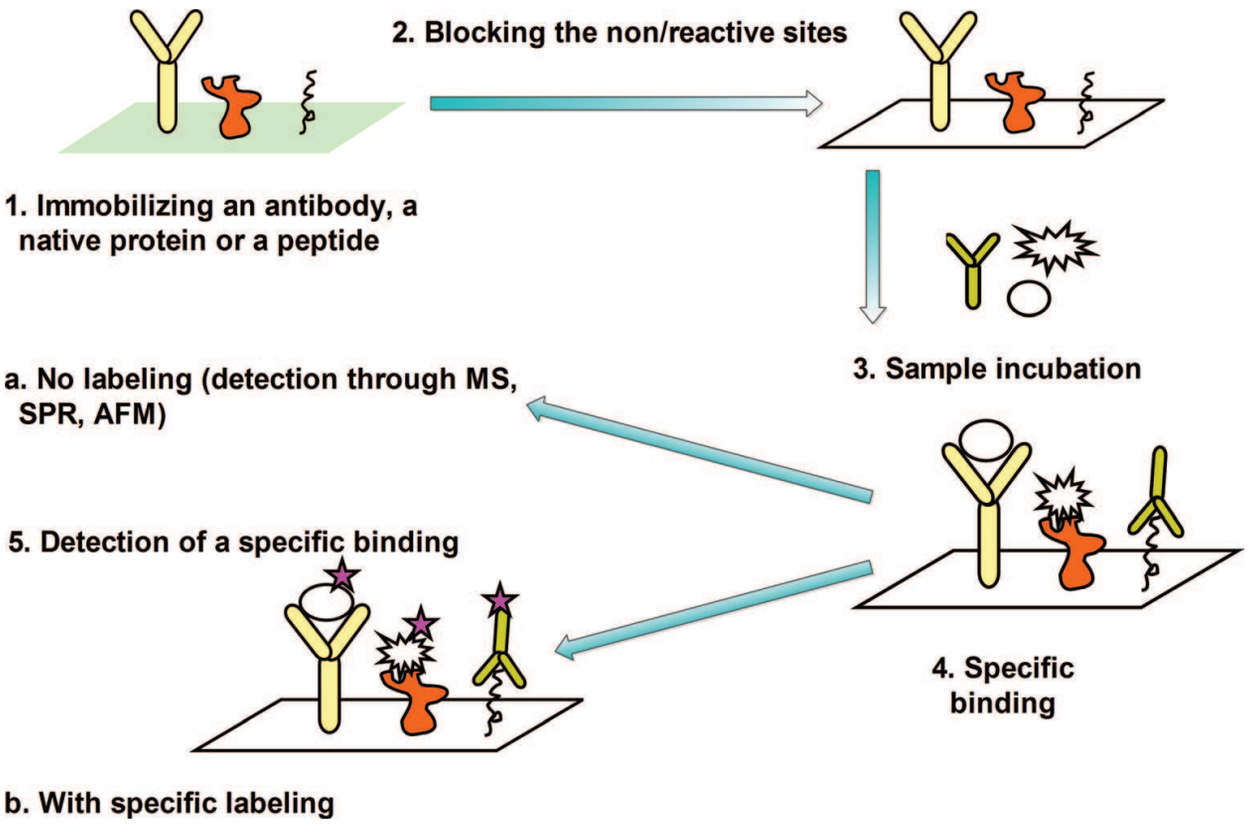


**Figure 2.** The general outline of any protein microarray work-flow. (1) Immobilizing an agent (*only for customized protein microarray slides*). (2) Blocking the non-reactive sites. (3) Sample incubation on specific slide. (4) The specific binding between an analyte from the sample and its corresponding antibody attached on the slide. (5) Detection step that can be without any labeling through e.g. MS (mass spectrometry), SPR (surface plasmon resonance), AFM (atomic force microscopy) (a) or with a fluorescent dye (b).

developed in order to discover new autoantibody targets, providing analysis for hundreds of samples and of their reactivity pattern against thousands of antigens simultaneously [73].

Customizing an array in relation to clinical purpose confirms the flexibility of these platforms in assisting molecular management of the disease. A customized platform was designed in order to monitor severe acute respiratory syndrome (SARS) infection by screening hundreds of sera based on reactivity against certain selected proteins from SARS coronavirus. Authors have reported that with this customized array, viral infection could be monitored for many months after infection [74]. This type of microarray platform has been further updated to a serological assay for the specific detection of IgM and IgG antibodies against the S1 receptor-binding subunit of the spike protein of emerging human coronavirus hCoV-EMC and SARS-CoV as antigens [75].

Protein microarray is a technology in continuous evolution offering multiple possibilities in updating other proteomic techniques. Therefore the development of the "microwestern array" is a clear proof how traditional methods like Western blot can be linked to novel technology, thus significantly expanding the research technological arsenal [76].

Data generated by WB require sometimes additional complementary proteomic methods to supplement and even support the scientific information. Such supportive task is often accomplished by protein microarrays providing important evidence on modulation of signaling networks and potential targets (or pathways); these factors or networks must first be identified, and array platforms allow exactly this development by exploring dozens of targets simultaneously within a single sample, providing lots of data which may be further investigated using traditional WB techniques [77].

As a conclusion, we present **Table 2**, with the advantages and disadvantages of each method.

## 7. Conclusions

The WB technique, as it first appeared, was an essential tool for demonstrating the presence of a protein in a biological sample. At first, it could be said to be an analytical and semi-quantitative method. Why semi-quantitative? Because not all of the protein in the gel passed to the membrane; either a small amount of protein remained in gel, or it was lost in the transfer buffer. With the development of powerful, automated equipment, especially with the emergence of the capillary system, this problem has been overcome. In addition, WB has become a multiplex. The modern capillary system has allowed several dozen samples to be analyzed at the same time, which is particularly important in clinical diagnosis. Moreover, with the emergence of increasingly performing devices, work time has been greatly reduced, and the reproducibility of the method has been achieved. By broadening the WB methodology, protein interrelations can now be analyzed, for example, by Far-Western blotting. We see that the old WB method has diversified over time and has been "emancipated" by development of more sophisticated technologies. It has become nearly as performing and sensitive as the other immunoassay methods described in this chapter: protein microarray, SPRi, Luminex or ELISA. Combining WB with other techniques in research is always a good solution.

However, performing tools are not always available to everyone. And then, we appeal to classical methodology, which sometimes means a true art for the one who applies it: it is necessary to avoid troubleshooting, safety measures must be taken—the presence of positive and negative controls, the use of good-quality antibodies, the antibody validation, comparison of the results by running multiple gels, and quantifying the amount of protein in the WB bands by measuring the optical density carefully and in more repetitions. Finally, data validation can also be done through at least one of the other immunoassay techniques already described. Of course, this is done with time-consuming manner, attention and scrupulousness, but the satisfaction of getting good results is always the same as Towbin and colleagues had when they put the WB technique in place.

As for the "dialog" between WB and other immunoassay techniques, we can say they complete and inspire each other. Thus, the microwestern array appeared as a combination of protein microarray and WB, enriching the research methodology in this direction. Also, validation by WB of antibodies used in other methodologies is a necessary step, which has, in fact, been used in many studies.

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## Author details

Emilia Manole[1,2,9]*, Ionela D. Popescu[1,6], Carolina Constantin[1,2,7], Simona Mihai[1,6], Gisela F. Gaina[1,3,8], Elena Codrici[1,6], Alexandra E. Bastian[4,5] and Monica T. Neagu[1,2,3,7]

*Address all correspondence to: emilia_manole@yahoo.com

1 National Institute of Pathology "Victor Babes", Bucharest, Romania

2 Colentina Clinical Hospital, Research Department, Bucharest, Romania

3 Faculty of Biology, University of Bucharest, Bucharest, Romania

4 Colentina Clinical Hospital, Pathology Department, Bucharest, Romania

5 University of Medicine "Carol Davila", Bucharest, Romania

6 Biochemistry Laboratory, Bucharest, Romania

7 Immunobiology Laboratory, Bucharest, Romania

8 Cellular Biology Laboratory, Bucharest, Romania

9 Molecular Biology Laboratory, Bucharest, Romania

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