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Chapter

Immunoassay Techniques Highlighting Biomarkers in Immunogenetic Diseases

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Abstract

Diagnosis of autoimmune diseases is crucial for the clinician and the patient alike. The immunoassay techniques most commonly used for this purpose are immunohistochemistry, ELISA, and Western blotting. For the detection of more specific biomarkers or the discovery of new ones for diagnostic purposes and as therapeutic targets, microarray techniques are increasingly used, for example, protein microarray, Luminex, and in recent years, surface plasmon resonance imaging. All of these technologies have undergone changes over time, making them easier to use. Similar technologies have been invented but responding to specific requirements for both diagnostic and research purposes. The goals are to study more analytes in the same sample, in a shorter time, and with increased accuracy. The reproducibility and reliability of the results are also a target pursued by manufacturers. In this chapter, we present these technologies and their utility in the diagnosis of immunogenetic diseases.

Keywords: immunoassay, protein biomarkers, autoimmune diseases, IHC, ELISA, WB, protein microarray, SPRi, Luminex

1. Introduction

An autoimmune pathology occurs when the immune system loses its ability to distinguish between its own cells and nonself cells, inducing the attack of self-tissue. This mechanism involves both the environmental factors and the genetic predisposition of the individual.

Proteomic technologies identify and separate different proteins of interest from biological samples, thus enabling their characterization as biomarkers, establishing their interactions, their role and the mechanisms in which they are involved, the identification of new diagnostic and therapeutic targets. The identification of protein biomarkers may be the basis for developing new methods of early diagnosis and treatment [1]. In general, an ideal biomarker should meet certain characteristics: be specific to a particular disease, be validated and confirmed as having specificity for that pathology, be able to early identify the disease, its testing to be easy and cheap as far as possible, reliable, and noninvasive [2, 3].

Although important advances have been made in deciphering immune function, the understanding of this function dysregulation and the specific autoimmune response remains limited. The domain is complex and includes, besides the disturbance of immune system functioning, gene alterations that regulate and control the self-tolerance. In this chapter, we will describe the techniques of highlighting the proteomic biomarkers involved in the pathogenesis of immunogenetic diseases.

In the case of immunogenetic diseases, one of the tissues that are first tested for specific biomarkers is blood, namely, the serum, which contains approximately 60–80 mg/mL proteins, besides amino acids, lipids, salts, and carbohydrates [4]. Applying proteomic immunoassay techniques for the diagnosis of immunogenetic diseases may also predict the course of disease, or result in a personalized treatment for patients [5, 6].

Proteomic biomarkers are particularly useful for providing the information on cellular signaling pathways, bringing early disease data, monitoring treatment response or adverse effects. They can be monitored from body fluids other than blood, such as: urine, saliva, cerebrospinal fluid and from different tissues (biopsies) [7].

The necessity to analyze very small amounts of proteins present in biological samples [8], 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. Immunohistochemistry, ELISA, and Western blotting are of the old methods that changed, adapted, modernized over time, but remained "on barricades" for protein biomarker investigation, especially in autoimmune disorders. 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. Next, the methods abovementioned are the other high sensitivity technics for validating proteomic biomarkers such as protein microarray, surface plasmon resonance, and Luminex multiplex assays. 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 (**Figure 1**).

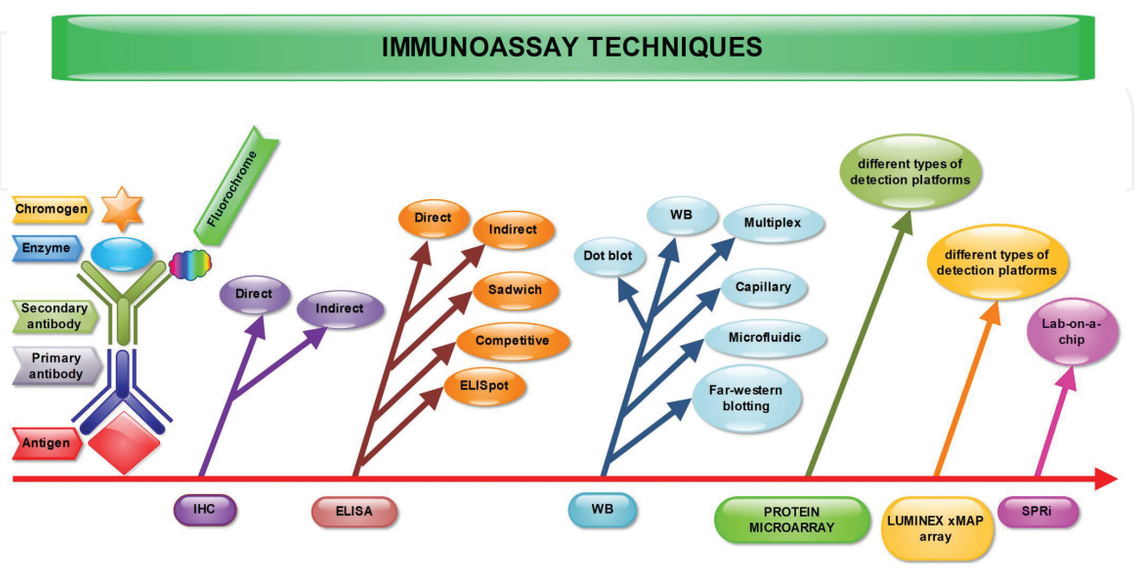


Figure 1.
The schematic representation of the immunoassay methods presented in this chapter, more or less in the order in which they appeared in time and how they evolved. These methods are based on the protein/antigen-antibody reaction that is shown on the left side—here is the indirect method: antigen → primary antibody → secondary antibody conjugated with a fluorochrome or an enzyme.

In many cases, the immunoassay techniques are used in conjunction for diagnostic, to confirm the presence of autoantibodies and then to characterize the expression of one or more specific biomarkers for a certain disease. More of this, these techniques can validate their mutual results.

2. Immunohistochemistry

The technique of immunohistochemistry (IHC) is a basic one, both in the anatomopathological diagnosis and in the research. It allows viewing of a protein of interest in a tissue section, specifying its location. This last aspect is very important and distinct IHC from other immunodetection techniques. The presence, reduction or the absence of the target protein allows a precise diagnosis or a personalized one. We do not intend to describe the technique itself, but we would like to mention it as the method of identifying immune antigens of interest, including immunogenetic diseases.

Based on the principle of the antigen-antibody reaction, this technique has undergone improvements over time. It started with a *direct IHC method*, the reaction antigen (target protein)-antibody, coupled with a fluorochrome. The first data on an attempt to use the direct IHC are from 1934 [9], but the use of fluorochrome for the first time was described in 1941 [10]. The introduction of an enzyme conjugated with an antibody and the visualization of the protein in light microscopy is due to Nakane and Pierce team [11]. The disadvantage of the IHC direct method is its low sensitivity.

Afterwards, an *IHC indirect method* was developed as follows: antigen-primary antibody, nonconjugated-secondary antibody (anti-primary antibody), conjugated with a fluorochrome or an enzyme, which convert a soluble substrate into an insoluble colored substrate [12, 13]. This method allowed the visual signal to be intensified.

The need to improve more the signal amplification has led to new changes. Thus the secondary antibody has been conjugated with other substances, such as biotin molecules, which in turn form complexes with streptavidin, forming a complex with an enzyme (e.g., horseradish peroxidase) [14]. More recently, an even more sensitive method was used in which a large number of secondary antibodies and enzymes are conjugated to a polymer chain (e.g., dextran) [15].

In the IHC technique, even *an array-like reaction* can be carried out on the same tissue section by targeting several proteins by using antibodies from different species (mouse, rabbit, goat, etc.), different enzymes coupled to the secondary antibody (e.g., horseradish peroxidase and alkaline phosphatase), different chromogens (e.g., 3,3′diaminobenzidine or 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium) or fluorochromes (e.g., FITC and rhodamine) with different colors.

Sometimes, especially when the protein of interest is low and the immunohistochemical signal is weak or with interruptions, a confirmation for protein expression by Western blotting is required. This confirmation is also required when we are not sure whether the antibody specifically binds to the protein of interest or if there is a nonspecific antibody labeling. The Western blot technique allows the identification of the protein as it is shown below.

3. Enzyme-linked immunosorbent assay (ELISA)

Old traditional ELISA technique was developed in 1971 by Engvall and Perlmann [16] and Van Weemen and Schuurs [17] and continues to be nowadays widely used

as a routine diagnostic method allowing quantitation of a large variety of proteins [18]. 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.

Direct ELISA, the simplest type of ELISA, could accurately quantify a specific molecule with high sensitivity from a wide variety of samples, and it is faster [19]. But the signal is less amplified.

Indirect ELISA detection is a two-step ELISA which involves a primary antibody and a labeled secondary antibody [20]. This method presents a higher sensitivity and flexibility (different primary detection antibodies can be used with a single labeled secondary antibody). The disadvantage is the occurrence of nonspecific signals.

Beside direct and indirect detection models, two other ELISA methods appeared, to avoid false positive or false negative results, with a high specificity, suitable for complex samples, with more sensitivity and flexibility: *sandwich ELISA* (quantify antigens between the two layers of antibodies) [21] and *competitive ELISA* (based on a competitive binding process between the original antigen in the sample and the add-in antigen, the more antigen in the sample, the less labeled antigen is retained in the well and the weaker the signal) [22].

Another ELISA method is *ELISpot assay*, widely used to evaluate an immune response, for example, in allergies or in autoimmunity [23, 24]. This technique, performed on PVDF membranes, has advantages like specificity, sensitivity, and wide range of detection.

However, the use of ELISA for assessing multiple analytes might be time consuming due to the large number of workflows occurring simultaneously. Moreover, ELISA is designed as a solid-state immunoassay, and the use of a planar matrix can restrict immunoassay capacity, sensitivity, and detection quality [25].

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 the interaction between several molecules within the inflammation milieu; therefore, studying the diseases necessitates a comprehensive perspective.

ELISA on a chip. In order to improve the method, in terms of using smaller quantities of samples, shortening the reaction time, avoiding sophisticated reading equipment, and reducing costs, a group of researchers tried to miniaturize the ELIZA platform [26]. They developed an ELIZA lab-on-a-chip system (ELIZA-LOC), which allows the use of only 5 μl of sample on a miniaturized 96-well plate combined with a CCD camera [27]. This system combines three functional elements: (i) reagent loading fluidics, (ii) assay and detection well plate, and (iii) reagent removal fluidics. The description of LOM technology (laminated object manufacturing) to obtain this system using polymer sheets was made by Rasooly et al. [28].

Besides miniaturization, another novelty is the washing step that is integrated directly in ELISA plate. The authors state that using this technology, there is no need for a specialized laboratory to perform the ELIZA test.

4. Western blot

The Western blot (WB), also known as immunoblot, is an analytical and quantitative technique for identifying specific proteins in many biological samples, liquid or tissue/cellular homogenates [29]. 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. The WB results can also guide us to a genetic investigation in case of partial deletion or duplication in the protein gene [30]. In addition, the WB method allows a quick comparison of target protein expression in many patients in medical diagnosis.

The WB technique was invented by Harry Towbin and co-workers 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" [31]. The WB name was given 2 years later by Neal Burnette, which also brought some improvements to this method, including the use of SDS-PAGE gels [32]. The name "Western" was inspired by the earlier name of other blotting methods, "Southern", named after the name of Edwin Southern, who published in 1975 a method for detecting specific DNA sequences [33], and "Northern" whose name was inspired by the name of the first blotting technique, "Southern", a RNA detection technique, developed in 1977 by Alwine et al. [34].

Over time, the method has improved and has become easier to achieve, with nearly all materials commercially available: transfer devices, antibodies, pre-casting 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 [35].

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 PVDF (polyvinylidene fluoride), that maintains the gel pattern as a copy [7]. 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, and radioisotope). The visualization is done colorimetric, by chemiluminescence, on X-ray film, or directly in the membrane with the aid of an imaging system.

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 ECL (enhanced *chemiluminescence kit*) for protein visualization by chemiluminescence can be reused. 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. For stripping, special buffers are using that can efficiently remove antibodies but do not remove too much amount of the proteins on the membrane.

The WB system size may vary, with electrophoresis/transfer tank, gels, and membrane: mini, midi, and large, depending on the investigated protein size and the time needed for separation. 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. New digital technologies offer a good and rapid bands visualization, avoiding underexposure or overexposure, as in the case of X-ray film developing. The images can be stored in a computer database and can be analyzed with software that measures the optical density of the bands.

4.1 Other methods based on the Western blotting technique

4.1.1 Multiplex Western blot

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. First Multiplex WB experiment (multiplex Western blot (MWB)) was optimized by Anderson and Davison [36] to study different muscle proteins involved in muscular dystrophies. This method allowed a simultaneously screening of multiple proteins with a different size on a pair of blots, using a cocktail of monoclonal antibodies which permitted the identification of primary deficient and second 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 in the upper part of the gel, with 5.5–4% polyacrylamide gradient, while proteins with molecular mass under 150 kDa are separated in the lower phase, 7% polyacrylamide gel.

Introduction of this technique has revolutionized the medical diagnosis and opened new perspectives in biomedical research. Simultaneous analysis of several proteins involved in different pathologies by MWB reduced the cost and time for analysis. By this method, it could be determined and compared proteins in the same sample as well as a secondary reduction of other proteins in a specific disease [37, 38].

4.1.2 Capillary electrophoresis and capillary Western blotting

By this method, the molecules are separated by the 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 classical 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 [35].

Capillary electrophoresis (CE) needs a smaller amount of sample than SDS-PAGE and offers a better resolution of a protein size. Proteins travel down the capillaries and are spaced according to the 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 nanoliters of the sample [39].

O'Neill et al. [40] 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 [40].

4.1.3 Microfluidic Western blotting

This technology reduces much more the amount of the sample required for WB and also the length of the capillaries from centimeters to microns, using microfluidic channels. He and Herr, in 2009, 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 in-line blotting membrane, and a high-efficiency identification of proteins of interest using antibody-functionalized membranes [41, 42]. The system requires only 0.01–0.5 μg of protein.

4.1.4 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 used for WB, or to evaluate the presence of a target protein in the sample before WB.

4.1.5 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 nonantibody 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 [43], receptor-ligand interactions, or screen libraries for interacting proteins.

5. Protein microarray

Protein microarray analysis has an increasingly use both for research purposes as well as 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 [44]. Thus, protein microarray is a proteomic tool that can deliver high-throughput data for revealing new therapeutic targets [45].

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 into 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.

Among all these variants, the antibody array type is preferred in tumor research domain or in biomarkers discovery/quantification due to technique's high versatility and reproducibility [46]. The reverse phase array format could also be used for biomarker discovery because it is specificity but has the disadvantage of being more laborious.

It is worth to emphasize 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 developed in order to discover new autoantibody targets, providing analysis for hundreds of samples and of their reactivity pattern against thousands of antigens simultaneously [47].

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 the 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 [48]. 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 [49].

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 [50].

Data generated by ELISA and 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 ELISA or WB techniques [51].

6. Luminex xMAP array

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 the configuration of various assays, quickly, cost effectively, and accurately, useful in clinical and research laboratories [52].

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 [53].

There are different types of advanced detection platforms (as depicted in **Table 1**), and 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 progres-sion monitoring and outcome prediction, even detecting major diseases in its earliest stages [54].

Technique	Advantages	Disadvantages	Use
IHC	Qualitative Fast Easy to detect Relatively inexpensive	Medium specificity Possible cross reactivity	Routine diagnstic tool Shows the localisation of antigen
ELISA	Quantitative High sensitivity Medium specificity Higher throughput than Western blot Automation potential	One protein/analyte at a time False positive results Labor intensive/time consuming/high reagent use Costly setup for automation	To quantify a single protein Confirmation of other screening method/validation
Western blot	High specificity High sensitivity Quantitative Automation potential	One or a small number of proteins/analytes at a time Labor intensive/ time consuming for the classical method. Resolved by the new equipments Qualitative, quantitative, especially with the newest equipments Difficult to transfer large or hydrophobic proteins—false negative results 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) Confirmation of other screening method/antibodies validation
Bead-based array (e.g., Luminex® technology)	High sensitivity High throughput and speed Multiplex and customizable panels of analytes Open-architecture design Low time, labor, and reagent use over traditional methods Versatility 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 Clinical implementation—available IVD kits
Protein microarray	High sensitivity Medium specificity Highest throughput 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

Technique	Advantages	Disadvantages	Use
SPRi	Very high sensitivity Label-free Real time method High throughput and speed Multiplex and customizable panels of analytes Open-architecture design Low time, labor, and reagent use over traditional methods Low time and reagent use Biochip reusable	High cost for a specialized equipment Suitable for liquid biological probes	To screen and quantify (quantitative) multiple protein interactions (and not only)/ panels of analytes, from the same sample Clinical implementation

Table 1.
Advantages and disadvantages of immunoassay methods presented in this chapter: IHC, ELISA, Western blot, bead-based array—Luminex technology, and chip-based array—protein microarray, SPRi.

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, 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 [55, 56].

Currently, Western blot is used to validate/confirm the identified biomarkers, and association between the xMAP technology and the 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, it was observed a superior sensitivity of Luminex xMAP compared with Western blot. Out of 87 confirmed HIV positive cases, Western blot confirmed 74.7% sensitivity, while Luminex xMAP identified 82.8% sensitivity ($p < 0.05$) [57]. 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 [58].

7. Surface plasmon resonance imaging, lab-on-a-chip

Since our goal is not describing surface plasmon resonance imaging (SPRi) methodology, we will not insist very much on the description of the technique. 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 [59]. The SPR immunoassay method is label-free (unlike ELISA); no label molecule is required for analyte detection [60]. 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 [61, 62], involving nucleic acid sequences [63–65], peptides [66], proteins [67, 68], and carbohydrates [69]. 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 nonlabeling and a real-time detection of biomarkers [70].

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. The main advantage of using SPRi in immunodiagnostics is the possibility of monitoring the antigen-antibody reaction in real-time, estimating kinetics, how quickly it occurs and how durable it is. In addition, it does not require any labels.

In comparison to ELISA and 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), and after washing the biochip, it is possible to immediately analyze another sample. SPRi takes less time than other methods. The disadvantage of SPRi would be that only liquid biological samples (blood, urine, cerebrospinal fluid, and cell culture medium) can be analyzed, and it does not analyze biological samples from different tissues/tissue lysates. 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.

As a conclusion regarding the technologies presented in this chapter, we show **Table 1** with the advantages and disadvantages of each method.

8. Immunoassay methods in immunogenetic disease diagnostic

Indirect immunofluorescence (IIF) for autoantibody analysis is one of the routine diagnostic methods. Different tissue sections or human tumor cell lines—HEp-2—are used as the source of antigen over which the serum of patients with specific autoantibodies is applied [71].

The antinuclear antibody (ANA) test is such a standard screening assay. The American College of Rheumatology declared HEp-2 IIF as the preferred method for ANA screening [72]. The large amount of ANAs can indicate an autoimmune disease, including systemic lupus erythematosus, Sjogren's syndrome, scleroderma, rheumatoid arthritis, idiopathic inflammatory myopathy, and others.

One of the immunohistochemistry method applications in autoimmune disease diagnosis is the detection of the presence of MHC I and, more recently, of MHC II in skeletal muscle of patients with idiopathic inflammatory myopathies (IIMs). It is a group of autoimmune systemic diseases, of which the most common forms are dermatomyositis, polymyositis, and inclusion body myositis. The study of muscle biopsy makes the difference in diagnosis between subtypes, but also among other types of myopathies and IIMs. In addition to other pathological features, the presence of MHC I and MHC II in sarcolemma gives the certainty of diagnosis, as long as they are not present in normal muscles [73–76]. Their overexpression in IIMs is induced by cytokines, including interferon and tumor necrosis factor alpha (TNF alpha) [77]. A study of 120 muscle biopsies from patients with different forms of IIMs showed a presence of MHC I in all biopsies and MHC II in 93% of them [76].

The MHC I expression appears early and precedes the lymphocyte infiltrate [78], persisting in late disease, and it is not attenuated by immunosuppressive treatment [79–81].

MHC II expression on antigen presenting cells activates T-helper cells and initiates an immune response without knowing the mechanism by which MHC II alleles mediate susceptibility to a given autoimmune disease [82].

From our experience in IIM cases where the IHC is not conclusive, a WB verification or validation is of great help in highlighting MHC I and II bands at their specific molecular weight.

From the more recent studies, we mention that the anti-signal recognition particle antibodies in the serum of IIM patients have diagnostic and prognostic value especially in the forms of immune-mediated necrotizing myopathy [83]. The authors draw attention to a mandatory IIF test along with the dot immunoassay to avoid false positive results from the latter method in pathologies not associated with IIM. The results sometimes depend on the nature of the antigen used in the technique and can be denatured.

ELISA is used as a diagnostic tool in autoimmune diseases, for evaluation of serum autoantibodies. Antinuclear antibodies (ANAs) directed against a variety of nuclear and cytoplasmic antigens are found with a high frequency in many systemic autoimmune disorders like systemic lupus erythematosus, scleroderma, Sjogren's syndrome, myositis, etc. *ANA-HEp-2 Screen ELISA* is an immunoassay method for the qualitative combined detection of IgG antibodies against human serum HEp-2 cells. Each well is coated with Hep-2 cellular lysate. The test detects in a well plate total ANAs against double stranded DNA, histone, SS-A (Ro), SS-B (La), Sm, snRNP/Sm, Scl-70, PM-Scl, Jo-1, and centromeric antigens.

HEp-2 cells allow the recognition of over 30 nuclear and cytoplasmic patterns that are given upwards of 50 different autoantibodies [84, 85]. The specificity of the test is closely related to the quality of the antigens used [86]. It is one of the most common methods of diagnosis in organ-specific autoimmune diseases, such as Grave's disease, primary biliary cirrhosis, insulin-dependent diabetes mellitus or systemic autoimmune disorders affecting different organs, such as systemic sclerosis, Sjogren's syndrome, and mixed connective tissue disease rheumatoid arthritis [87, 88].

From recent research studies [89], we want to mention cortactin antibodies as new biomarkers in double seronegative myasthenia gravis (myasthenia gravis form dSNMG). ELISA tests validated by WB have demonstrated that the presence of cortactin autoantibodies is a biomarker to be taken into account, suggesting that the disease will be ocular or mild generalized and could be done routinely in the future.

Another work on rheumatoid arthritis shows that, apart from the autoantibody system that recognizes citrullinated proteins, the identification of another antibody system against carbamylated proteins has an important early diagnostic value, predicting a more severe course of disease [90]. The ELISA method used in this study could become routine for serum testing of patients with rheumatoid arthritis.

Western blot. To highlight the importance of WB technique in clinical diagnosis, we give some eloquent examples below. The WB method has been used in many studies, along with immunoprecipitation, ELISA, and flow cytometry, to demonstrate the quantitative or qualitative modification of proteins of interest in autoimmune diseases in order to find new biomarkers or therapeutic targets. WB has proven to be a good tool for serological tests.

Line-blot immunoassay is a Western blotting method that uses recombinant antigens immobilized on straight lines on a nylon strip. They are incubated with patient serum containing autoantibodies. They bind to the antigens present on the strip and are viewed colorimetrically. Interpretation of the results is done by comparing the color intensity of strips with the color of strips of a positive standard. There are some studies that have shown the utility of this method in the detection of autoantibodies present in serum but which could not be identified by IIF, for example, anti-SS-A/Ro in Sjogren's syndrome [91, 92].

Haroon et al. have demonstrated using the WB method that there is an interaction between endoplasmic reticulum aminopeptidase 1 (ERAP1) with human

leucocyte antigen (HLA)-B *27 in ankylosing spondylitis [93], and that the HLA-B27 molecules could alter the ERAP1 level. The functional interaction between ERAP1-peptide and HLA-B27 could thus be the missing link in the pathogenesis of ankylosing spondylitis.

Stagakis et al. studied whether anti-TNF therapy improves insulin resistance in rheumatoid arthritis [94]. Western blot was used to analyze the proteins p-Ser^{312} IRS-1 and p-AKT from peripheral blood mononuclear cell lysates. It has been established that anti-TNF alpha therapy has a positive effect, improving insulin sensitivity and reversing the defects in signaling insulin cascade in this disease.

Tsui et al. have conducted a study of the serum levels of noggin (NOG) and sclerostin (SOST) in patients with ankylosing spondylitis, more specifically, on the immune response to these two molecules [95]. The WB method was used to quantify the relative amounts of NOG/SOST-IgG immune complexes. An increased level of NOG/SOST-IgG immune complexes was found in patients with this pathology.

Rizzo et al. [96] showed that the dimeric form of the HLA-G molecule is associated with the response to methotrexate treatment in patients with early rheumatoid arthritis. HLA-G dimeric and monomeric forms have been highlighted by WB. The presence of dimeric form in plasma prior to methotrexate therapy could be a biomarker for the patient's response to treatment.

Protein microarray. Antibody microarrays could provide a real-time vision of biological processes, such proteomic instrument being used in clinic to analyze serum and plasma in several pathologies including autoimmune disorders. One of the autoimmune diseases approached through protein array is *systemic lupus erythematosus* (SLE) where SLE clinical heterogeneity and the absence of robust biomarkers to evaluate the disease states and differentiate from other autoimmune conditions are yet to be resolved [97]. Thus, using an antibody-based leukocyte-capture microarray, mononuclear cells isolated from peripheral blood of 60 SLE patients were processed for obtaining proteomic patterns to distinguish SLE from healthy subjects. With this array platform, it was improved the conventional SLE diagnostics and disease states elucidation [98]. Moreover, an "in-house" antibody microarray comprising 135 human recombinant single-chain fragment variables aiming various immune proteins were used to examine *systemic sclerosis* (SSc) and SLE patients. This tailored array identified a significant number of differentially expressed proteins that delineate SLE from systemic sclerosis, thus surpassing disease classification through conventional clinical parameters, including, ANA, anti-DNA, SLEDAI-2 k, C1q, C3, C4, and CRP [99].

Another challenging field for protein array is related to *rheumatoid arthritis* (RA) as it could detect biomarkers specific for arthritis and not for autoimmune diseases in general [100]. Some research groups have started to develop different antigen arrays for differential diagnosis and even RA molecular classifying. Panels of proteins were detected, among these three proteins, namely, WIBG, GABARALP2, and ZNF706, were suggested as potential specific markers for RA early stages [101]. Hence, protein arrays bring valuable data to immune-disease background allowing exploration of numerous samples in parallel and thousands of targets.

Antibodies against ion channels, receptors, synaptic proteins, etc. confirm protein microarrays as a future potential tool in routine diagnosis [102]. Whatever commercially available or customized platforms, antibody arrays start to emerge in clinic by designing *omics* disease signatures helping the disease management.

The protein microarray was used in a study of *pemphigus vulgaris*, an autoimmune skin disease, to identify the entire set of antibodies, bringing extra data about the complex relationship between genetics and disease development [103]. The results were correlated with those obtained by the ELISA and proved to be compatible. The main targets for autoantibodies are desmoglein-3 and 1, but the

study showed that there are autoantibodies that are not directed to desmoglein at a significant number of patients.

A study regarding *ankylosing spondylitis* using the protein microarray, confirmed by ELISA, to characterize anti-ankylosing spondylitis autoantibodies, showed that anti-protein phosphatase 1A (PPM1A) autoantibodies are present in the serum of the patients and that they could serve not only as biomarkers for diagnosis, disease severity, and response to anti-TNF therapy, but also as a therapeutic target [104].

Luminex xMAP technology has developed as an alternative to planar microarray methods. Bead-based immunoassays are one of them. The determinations by this method and by ELISA of anti-thyroid peroxidase and anti-thyroglobulin antibodies in autoimmune diseases have been shown to be compatibles [105].

There is a commercially available kit for ANA detection, which is low cost and saves time. Antigens corresponding to autoantibodies are linked to polystyrene microspheres labeled internally with different amounts of two different fluorochromes, resulting in 100 different color spectra. Each microbead carries an antigen specific for a single antibody [106].

Good results were obtained in assessing a number of antinuclear autoantibodies as: dsDNA, Sm and Sm/RNP (in systemic lupus erythematosus), SS-A/Ro and SS-B/La (in Sjogren's syndrome), Jo-1 (myositis), ribosome (systemic lupus erythematosus), and centromere (systemic sclerosis) [107].

One of the problems with this technology could be the lack of a true quantitative calibration due to the difference in affinity of the antibodies for the antigen [108]. Some authors argue that it is also necessary to validate the results by other immunoassay methods [106], while others claim that the accuracy of the technique is similar to that observed by ELISA [109, 110].

There are studies in which the Luminex methodology is used for the analysis of serum biomarkers in various autoimmune diseases. Thus, in an article on ankylosing spondylitis, certain cytokines as hepatocyte growth factor (HGF), CXCL8, and matrix metalloproteinases (MMP-8 and MMP-9) identified from a large number of biomarkers by Luminex could be diagnostic targets, their serum levels being increased in this disease [111].

In other chapter regarding ankylosing spondylitis, Luminex bead-based technology was used for serum cytokines analysis, and the conclusion was that the utilization of TNF alpha inhibitors decreases the number of T cells producing proinflammatory cytokines [112].

Mou et al. showed, using Luminex technology in combination with PCR, that in ankylosing spondylitis patients from Southern China with HLA-B27 in their serum, HLA-B2704 subtype predominates. And the HLA-B2715 subtype may have a disease prognostic value, early onset being related to this subtype [113].

Surface plasmon resonance imaging. Despite its great sensitivity, this technology is relatively little used to determine the concentration of some analytes. Improving signal amplification methods is one of the research goals in this technique.

In some autoimmune diseases, such as rheumatoid arthritis (RA), psoriatic arthritis, systemic lupus erythematosus, or Sjogren's syndrome, autoantibodies attack citrullinated proteins, and the presence of anti-citrullinated proteins, antibodies is a standard test in these cases. The use of SPRi for monitoring autoantibodies that bind to different citrullinated targets was first described by Lokate et al. SPRi has shown its ability to detect the interaction between citrullinated peptides and serum autoantibodies in RA patients in one step [114].

SPRi microarray technique was also used in a more recent research to identify autoantibodies against citrullinated protein (ACPA) profiles in patients with early onset rheumatoid arthritis. The authors made a comparative study using citrullinated

and noncitrullinated peptides [115]. The study showed that SPRi is a suitable methodology for detecting ACPAs in the serum of patients with this pathology.

A subsequent study was also revealed by SPRi, the presence of citrullinated B-cell epitopes in fibrinogen [116].

A research team [117] showed the use of SPRi and gold particles to amplify the signal for the detection of inflammation biomarker TNF-alpha in serum. Also, the use of a specific buffer solution for sample dilution was utilized to reduce the nonspecific binding in real samples. Thus, a low limit of detection, as well as a good reproducibility and the longevity of chips, is a good motivation to use this immunoassay method to detect biomarkers that are in low concentrations in biological samples.

Buhl et al. reported in a research paper the use of SPRi technology for the anti-dsDNA detection in systemic lupus erythematosus [118].

9. Conclusions

Immunoassay methods have many advantages but some limitations too. Their importance in identifying different biomarkers for diagnosis or personalized therapy is essential. That is why they have diversified so much, in order to be able to answer all the challenges. Additionally, these methods and technologies have also specialized in an advanced degree, so that they can detect smaller amounts of molecules with as high a precision as possible in a shorter time. The antigen-antibody response gives them great sensitivity. The development of more advanced equipment leads to the automation of these methods and to a greater efficiency, with applicability in diagnosis and therapeutic monitoring, in discovery of new biomarkers and even in pharmacology.

In order to be used for diagnosis in different laboratories, these methods and kits should be standardized. The problems to be posed are: the clinical manifestation of the disease in different individuals, the source of the antigen, the specificity and sensitivity of the autoantibodies for different antigens, the reproducibility of the assay, and the precision and the accuracy of the method [91, 119, 120].

Some studies show a good correlation between IIF and ELISA methods [84, 121, 122], and others, on the contrary, show different results between these methods [71, 123].

Multiplex technologies are gaining more and more followers in recent years by allowing simultaneous analysis of a multitude of analysts, saving time and costs. However, there are studies showing that compared to the old methods, some false negative or false positive results are obtained [124–128]. Cross reactivities may also occur [129].

Assay kits produced by different manufacturers can show variable results also. More than this, the methodology used by each laboratory can lead to different results, even by using the same kit. International standardization is required. A collaboration between an international body and organizations responsible for quality of assessment of assays is desirable, so that a collaboration among clinicians, diagnostic laboratories, and manufacturers to be established.

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