

Review

Protein biochip systems for the clinical laboratory

Anne Marie Dupuy^{1,*}, Sylvain Lehmann^{1,2} and Jean Paul Cristol¹

¹ Department of Biochemistry, Lapeyronie and St Eloi Hospital, Montpellier, France

² Institute of Human Genetics, CNRS UPR1142, Montpellier, France

Abstract

Classical methods of protein analysis such as electrophoresis, ELISA and liquid chromatography are generally time-consuming, labor-intensive and lack high-throughput capacity. In addition, all existing methods used to measure proteins necessitate multiple division of the original sample and individual tests carried out for each substance, with an associated cost for each test. The chip system allows several tests to be performed simultaneously without dividing the original patient sample. This system facilitates the development of multiplexed assays that simultaneously measure many different analytes in a small sample volume. These emerging technologies fall into two categories: 1) spotted array-based tools, and 2) microfluidic-based tools. Miniaturized and multiplexed immunoassays allow a great deal of information to be obtained from a single sample. These analytical systems are referred to as “lab-on-a-chip” devices. This article presents current trends and advances in miniaturized multiplexed immunoassay technologies, reviewing different systems from research to point-of-care assays. We focus on a subset of chip-based assays that may be used in a clinical laboratory and are directly applicable for biomedical diagnosis. Recent advances in biochip assays combine the power of miniaturization, microfluidics, micro- to nanoparticles, and quantification. A number of applications are just beginning to be explored. The power of biochip assays offers great promise for point-of-care clinical testing and monitoring of many important analytes.

Keywords: biochip; clinical applications; miniaturization; multiplexing.

Introduction

Clinical proteomics is an exciting new sub-discipline of clinical biochemistry that involves bedside appli-

cation of proteomic technologies. Classical methods of protein analysis such as electrophoresis, ELISA and liquid chromatography are generally time-consuming, labor-intensive and lack high-throughput capacity. In addition, all existing methods used to measure proteins necessitate multiple division of the original sample and individual tests carried out for each substance, with an associated cost for each test. The protein chip system is the next generation of chip technology that can be used as an important tool for proteomics research. The chip system allows all tests to be performed simultaneously without dividing the original patient sample; it is therefore faster (few minutes vs. hours), more convenient (one experiment to obtain data for multiple analytes) and less expensive (three-fold) than conventional methods, in particular ELISA-based technologies.

Different methods are used to immobilize proteins on chips and to detect protein binding with a sensor. This type of system facilitates the development of multiplexed assays that simultaneously measure many different analytes in a small sample volume. It can be used for a wide range of applications and has enormous potential for diagnosis. These emerging technologies fall into two categories: 1) spotted array-based tools, and 2) microfluidic-based tools. Miniaturized and multiplexed immunoassays improve throughput by greatly increasing the amount of information obtained from a single experiment.

Microfabricated analytical devices that simultaneously integrate multiple determinations with the actual measurement step are of considerable interest. These analytical systems are referred to as “lab-on-a-chip” devices. This article presents current trends and advances in miniaturized multiplexed immunoassay technologies from the point of view of: 1) research vs. in vitro diagnostics (IVD) medical devices; 2) routine vs. specialty testing in the clinical laboratory; and 3) central laboratory vs. point-of-care (POC) testing. We focus on a subset of chip-based assays that may be used in a clinical laboratory and are directly applicable for biomedical diagnosis.

General characteristics of chip-based assays

Miniaturization

Microfabrication technology has advantages and limitations. It has the potential to improve the development, automation, cost reduction and throughput of various proteomics approaches. In addition, miniaturization reduces the amount of analyte required, and can potentially provide low-cost disposable chips for a variety of applications.

*Corresponding author: Anne Marie Dupuy, Laboratoire de Biochimie, Hôpital Lapeyronie, 191 Avenue du Doyen Gaston Giraud, 34295 Montpellier cedex 5, France
Phone: +33-4-67338315, Fax: +33-4-67338393,
E-mail: am-dupuy@chu-montpellier.fr

In spite of these advantages, the biochip system has its limitations. These include multiple-based reactions associated with low specificity, high evaporation rates, and possible cross-reactions. Among the other limitations of biochips, the dynamic range is an important issue for measurement of low- and high-abundance proteins at the same time. This aspect makes the problem of cross-reactions even more challenging. Even though antibodies are known to bind specifically to their antigen, assay development usually reveals non-specific binding to other antigens and antibodies. The affinity of these binding events may be comparatively low but may cause problems if low- and high-abundance proteins are determined in parallel.

The surface topography is critical to the success of chips as a diagnostic technology platform. Strict quality control procedures need to be in place during the chip manufacture. The biochip surface must be chemically inert and compatible with available detection methods.

Detection strategy

Current determinations have evolved from immunoassays, radioimmunoassays and ELISA assays (1). Labeled probe detection methods differ, based on the immobilized bait molecule. Currently the most important approaches are competitive, sandwich and antibody capture methods.

Competitive or direct detection strategies use a labeled antibody to directly bind to the target molecule immobilized on a substratum. Amplification strategies based on avidin-biotin binding enhance the sensitivity of detection. The advantages of this approach are that only one incubation step is necessary and that the "prozone effect" at high analyte concentrations cannot occur. The disadvantages are that the concentration range over which the analyte can be quantified without sample dilution is rather narrow and that the antigen or antibody produces the same response, and therefore cannot be distinguished in a one-step assay.

Sandwich methods require two distinct antibodies for detection of a capture molecule. The first antibody is immobilized on the substratum and the molecule of interest binds to its known antibody. A second labeled antibody detects the bound antibody-capture molecule complex. In principle, quantification can be achieved over an extremely wide analyte concentration range using such sandwich methods. The "prozone effect" can be avoided in the following ways: (i) using sequential incubation steps for samples and labeling; or (ii) using monoclonal antibodies. Rheumatoid factor (RF) is known to be a potentially interfering factor.

Antibody capture methods or indirect detection strategies use an immobilized antibody for capturing labeled specific molecules from a complex mixture of query molecules.

It is expected that competitive methods will decrease in importance, and that sandwich and antibody capture methods will be the main assay principles used in biochip technology.

Labeled probe methodology

Labeled probe detection methods have evolved from clinical immunoassay protocols, incorporating chromogenic, chemiluminescence, fluorescence, and radioactive labeling strategies for the detection of immobilized targets.

The selection of a substratum is the first consideration in selecting a detection method. The ideal substratum has common attributes that enhance the signal-to-noise ratio of the analyte: minimal autofluorescence, limited non-specific binding, high surface area-to-volume ratio, inert to biological molecules and compatibility with available detection methods.

Microporous surfaces and membranes such as nitrocellulose, nylon and polyvinylidene difluoride (PVDF) have high surface area-to-volume ratios, high binding capacity and are easy to use, making them amenable to protein microarrays.

Technical characteristics

There are seven considerations that should be assessed in evaluating quantitative assays: specificity, sensitivity, practicality, reliability, multiplexing capabilities, cost and time.

Higher specificity can be obtained by avoiding the presence of any antibody in the assay system with specific reactivity against non-analyte epitopes, and by selecting combinations of monoclonal antibodies that may further increase specificity.

Higher sensitivity can be obtained either by selection of antibodies with an extremely high affinity, or by reduction of the height and variability of the background reaction, which makes very low analyte concentrations more readily detectable.

Higher practicality has some evident advantages but can lead to reduced specificity and sensitivity. The simplicity of these types of assays lies in the simultaneous incubation of label, solid phase and sample without the risk of any "prozone effect" and eliminating the washing steps.

Each manufacturer of a biochip assay must be in a position to release coefficients of variation for inter- and intra-series assays, as well as for inter-laboratory assays, because reliability is clearly a guarantee of quality.

Multiplexing capacity is clearly an advantage, because this methodology requires a low volume of sample for data on multiple analytes, which is extremely useful for the diagnosis and monitoring of diseases.

Miniaturization of biochips to sub-microliter volume for the quantification of many proteins in a single experiment is less expensive than ELISAs, as it reduces the amount of material and reagents required.

High throughput is increasingly required in the clinical laboratory, particularly in diagnostic testing.

Current applications

There are many clinical applications and options available with biochip array technology, including allergy testing, autoimmune disease, cancer markers,

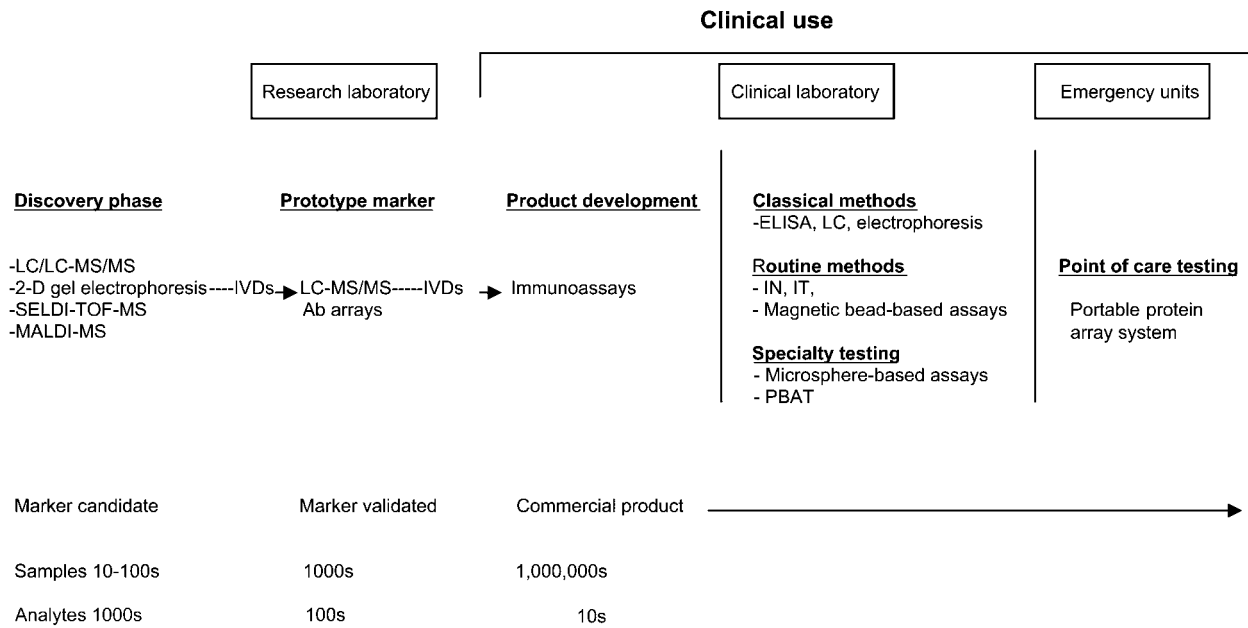


Figure 1 Schematic diagram of a three-step diagnostic system exploiting different technologies in each stage, in accordance with different localizations. LC, liquid chromatography; MS, mass spectrometry; SELDI, surface-enhanced laser desorption/ionization; TOF, time of flight; MALDI, matrix-assisted laser desorption/ionization; IVD, in vitro diagnostics; Ab, antibodies; IN, immunonephelometry; IT, immunoturbidimetry; PBAT, protein biochip array technology.

cardiac markers, metabolic and endocrine markers, kinase/phosphorylated protein, drug abuse panels and cytokines. Because antibody pairs are readily available, most applications so far have focused on cytokines and chemokines. The real limitation of any biochip assay lies in the utility and quality once there is sufficient specificity and high antibody affinity.

Different systems from research to point-of-care assays

Research vs. in vitro diagnostic medical devices

Proteomics research has allowed the potential discovery of thousands of intact and cleaved proteins in the serum proteome. Diagnosis using disease-related protein requires the separation and identification of protein biomarkers. To be effective, a clinically useful biomarker should be measurable in an accessible body fluid such as plasma, urine or saliva using clinical tests. These clinical tests are so-called in vitro diagnostics (IVDs). As recently described by Zolg and Langen (2), there are three distinct development phases from the identification of a protein to the launch of a diagnostic product in an immunoassay format: the discovery phase, in which a variety of proteomics technologies are applied to identify marker candidates; prototype development, in which immunological assays are established and validated in defined sample collectives; and the product development phase, in which assay formats suitable for automated platforms are realized (Figure 1). IVD devices include all the reagents, reagent products and calibrator materials, as well as the instrument, for use in the in vitro examination of human tissue, blood or fluid samples to provide information about a patient's state of health (3). IVD devices or laboratory tests require

review by the Food and Drug Administration (FDA) in the US before being marketed to ensure appropriate performance and labeling (4, 5). These submissions are referred to as 510(k)s or premarket approval applications (PMAs) and encompass established administration procedures. IVDs developed as in-house, or so-called "home-brew", tests or laboratory test services are considered medical devices. The FDA recently established a new regulatory paradigm for in-house tests based on the classification of the active ingredients or building blocks of these tests as analyte specific reagents (ASRs). ASRs are exempt from pre-market review but are subject to manufacturing and labeling conditions. IVDs are classified according to the risk of the controls required: class I (low risk), class II (moderate risk), and class III (high risk) products. Class I products are occasionally reviewed as 510(k)s, class II products are subject to 510(k) review and class III products are subject to PMAs. To meet IVD requirements, the following are essential: (i) diagnostic tests that allow for definite and reliable diagnosis tied to a decision on interventions; (ii) performance characteristics (in particular test accuracy) for each analyte; and (iii) adequate diagnostic accuracy, including sensitivity and specificity as determined by positive or negative predictive values. These IVD requirements reflect features that are useful in clinical practice (6). Antibody arrays are likely to represent the most practical approach for the identification, quantification and functional analysis of proteins in basic and applied proteome research.

Microarray technology is applicable for studying protein-protein, protein-ligand, kinase activity and post-translational modifications of proteins. The development of protein arrays allowing the detection of 10–10,000 proteins in the same sample is technically very difficult because of the structural diversity and

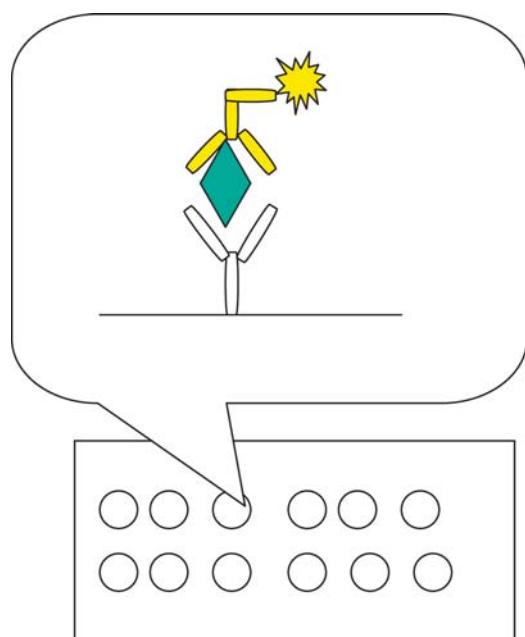


Figure 2 Protein microarray technology. Known antibodies are arrayed and used to capture antigens from unknown samples. The antigen either needs to be labeled directly with a fluorophore or a second binder can be used.

complexity of proteins. The aim of manufacturers is to develop and apply a technology based on protein microarrays for high-throughput detection, identification and quantification of proteins of interest.

These emerging technologies fall into two categories: 1) spotted array-based tools; and 2) microfluidic-based tools. The first objective of these technologies is to profile various serum proteins to identify novel biomarkers and potential drug targets. An alternative approach, using well-characterized antibodies, permits a simple array to detect the expression levels of multiple proteins in a sample.

Spotted array-based tools and clinical applications

In most cases, protein biochips are composed of arrays of immobilized proteins or ligands selected against known analytes. The solid-liquid interface used generally involves physical adhesion, chemical cross-linking or biomolecular affinity capture. The chip surface material and the method for protein deposition will have a profound influence on the overall efficiency of protein immobilization and the preservation of protein activity. Proteins have been successfully immobilized on nitrocellulose (7), aldehyde-treated glass (8), epoxy-derivatized slides (9), BSA-N-hydroxysuccinimide-treated slides (10), silanized glass slides (11) and nickel-coated slides (8). These are usually based on chips containing immobilized capture agents.

Protein biochips come in a wide array of formats, including:

- Antibody arrays to detect antigens from cell lysates, body fluids or cell culture supernatants;
- Tissue extract or purified antigen arrays to detect serum antibodies or known proteins with detection antibodies;

- Bait proteins immobilized to detect interacting proteins for pathway identification studies;
- On-chip activity profiling of immobilized enzymes; and
- Large-scale proteome arrays of entire expression libraries encoded with fusion proteins as immobilization tags.

We focus on simple arrays that can be directly used for clinical applications (Figure 2). The development of arrays that will be useful in the clinical laboratory depends, in part, on the stage of development of antibodies for the proteins of interest. Research laboratories will, however, be involved in much of the bio-technical development necessary before commercially supplied biochips become available. A number of groups, either leaders in protein profiling biochips or in ELISA techniques, are starting to manufacture simple protein arrays to measure samples for known markers. An example is the RayBio kit (RayBio® Cytokine Antibody Array, Norcross, GA, USA) which uses a simple array format to simultaneously detect up to 126 cytokine expression levels from serum samples, supernatants and cell lysate samples. This membrane-based assay uses specific cytokine antibodies spotted on an array membrane. The RayBio protein arrays include general cytokine arrays and specific arrays for angiogenesis, inflammation and atherosclerosis profiling factors. The Tebu-bio Laboratories platform works in association with RayBio to offer a custom testing service. After sample incubation and washing steps, a cocktail of biotinylated antibodies is added to the membrane. Streptavidin-peroxidase is then added, followed by a chemiluminescent substrate. The signal is detected either with a chemiluminescence device, or after film exposure. This new protein array system offers several advantages to the clinical biologist: no special equipment is required, the high sensitivity detects most proteins at pg levels, volume as little as 100 μ L of sample is required to detect over 120 cytokines, and the system is easy to use with minimal experience and high specificity.

The technique involving so-called abundance-based microarrays allows measurement of the abundance of specific biomolecules using ASRs. This technique has two limitations: it requires two ASRs for each target, and multiplexing is more difficult. The use of two ASRs recognizing different epitopes of the same analyte, such as in a sandwich immunoassay, avoids the need for labeling and yields a highly specific signal. However, this approach is less convenient for simultaneous assessment of many analytes and requires the existence of two independent ASRs for each analyte. A useful hybrid approach is to print multiple different capture ASRs for the same analyte on different features on the array and to carry out detection using the labeling approach. Concordant signals from the multiple ASRs confirm the specificity of the signal, while still allowing simultaneous measurement of multiple analytes (12).

Microfluidic-based tools and clinical applications

Microfluidic chip-based immunoassays represent one

of the most important innovations in biochip technology. Microfluidic devices offer unique advantages in sample handling, reagent mixing, separation and detection.

This technology is based on devices and processes that handle volumes of liquid on the nanoliter, or even picoliter scale. The silicon, glass or plastic devices have overall dimensions of a few millimeters; fluids flow through micropore channels produced by photolithographic etching or other high-precision techniques. Since microfluidic chips can be combined with mass spectrometric analysis, a microfluidic device has been devised in which an electrospray interface to a mass spectrometer is integrated with a capillary electrophoresis channel, an injector, and a protein digestion bed on a monolithic substrate. This chip thus provides a convenient platform for automated sample processing in proteomics.

A microfluidic device can be identified by the fact that it has one or more channels with at least one dimension of less than 1 mm. Common fluids used in microfluidic devices include whole blood samples, bacterial cell suspensions, protein or antibody solutions, and various buffers. Microfluidic devices can be used to obtain a variety of interesting measurements, including molecular diffusion coefficients (13), fluid viscosity (14), pH (15), chemical binding coefficients (13) and enzyme reaction kinetics (16). Other applications for microfluidic devices include capillary electrophoresis (17), isoelectric focusing (18), immunoassays (19), flow cytometry (20), sample injection of proteins for analysis via mass spectrometry (21), PCR amplification (22), DNA analysis (23), cell manipulation (24), cell separation (25), cell patterning (26) and chemical gradient formation (27). Many of these applications can be utilized in clinical diagnostics (28, 29).

The strategy of two leaders, Biosite (San Diego, CA, USA) and Zyomyx (Hayward, CA, USA) in microfluidic protein chip technology is to expand the commercial potential of different products. Both have launched protein profiling biochip systems and clinical application systems. Zyomyx developed the Zyomyx[®] Human Cytokine Biochip. Each biochip contains six sample channels and supports the analysis of 30 distinct and validated cytokine assays. Zyomyx[®] Cytokine Biochips are ready to use, fully validated, multiplexed protein quantification assays that use the Zyomyx[®] Protein Profiling Biochip system.

Biosite was the first company to commercialize Microfluidic Chip-Based Immunoassays offered for use in clinical applications. Biosite's protein array comprises two components: a protein array device, and the Triage protein. In 2001, Biosite manufactured the Triage protein chip, which simultaneously measures 100 different proteins on one array from as little as 0.05 μ L of plasma or urine or 0.1 μ L of whole blood. This technology could be applicable to large-scale quantification and profiling of large numbers of proteins. In the section on Central laboratory vs. point-of-care testing, we describe the Biosite technology, which is the most common in emergency units in the shape of the Triage MeterPlus System.

The Biosite patent encompasses: 1) sample handling; 2) microcapillary fluidics; 3) high-sensitivity fluorescence energy transfer dyes; and 4) antibody development. In fact, patent issues are of major importance in the area of biochips, in particular with respect to the use of reagents and distinct analytes for diagnostic purposes.

- 1) Sample handling: Developments relating to sample handling and preparation allow whole-blood to be passively separated into its plasma component or to be passively lysed to release the target analyte. Biosite has developed technologies for the handling of stool samples that concentrate and purify the target analytes or organisms from solid stool materials. In addition, Biosite has developed technologies that can be used to test urine samples.
- 2) Microcapillary fluidics: This technology involves microcapillaries that control the flow of fluids in immunoassay processes. The protein array format uses several different microcapillary designs to control the contact of samples with reagents and to control the flow of fluid throughout the protein array. When a sample is added to the protein array, a filter contained within the array separates blood cells from plasma, which is then directed by capillary forces into a chamber that contains dried immunoassay reagents. After an incubation time that is determined by another microcapillary element of the array, the volume of the sample that has contacted the reagent flows down a capillary path that brings it into contact with an antibody array. Biosite has also developed the engineering capability to design unique microcapillary structures in plastic parts and to manufacture them in commercial-scale quantities using injection molding processes.
- 3) High-sensitivity fluorescence energy transfer dyes: Biosite has invented their own proprietary dyes, which satisfy three criteria: (a) they are excited at near-infrared wavelengths, and therefore are usable with complex biological samples such as serum, plasma, and whole blood; (b) they are stable for the dating period of the product; and (c) they show no overlap between emission and excitation, so that detection can be performed with inexpensive solid-state components.
- 4) Antibody development: These capabilities allow rapid identification and development of antibodies with optimal specificity, affinity, and stability characteristics.

The benefits of Microfluidic Chip-Based Immunoassays include portability and a number of significant other advantages. First, because the volume of fluid within these channels is very small, usually several nanoliters, the amount of reagents and analytes used is quite small. This is especially significant for expensive reagents. Easy calibration, speed, high sensitivity and one-step operation for whole blood (or any other fluid) are the main advantages of microfluidic systems. The fabrication techniques used to construct microfluidic devices are relatively inexpensive and

are very amenable to both highly elaborate, multiplexed devices and to mass production. Microfluidic technology allows the fabrication of highly integrated devices for performing several different functions on the same substrate chip. In addition, up to 100 proteins can be simultaneously measured. Microfluidic immunoassays appear likely to reach POC testing first.

The miniaturization of a sandwich immunoassay to a 20- μm -wide area on a surface harbors significant risks. Defects such as surface inhomogeneities, dust particles, uneven flow of solution, uncontrolled evaporation, air traps, depletion of reactants and cross-contamination can compromise the assay in one or several areas (30). Different methods can be used to move liquids in microfluidics, such as pressure-driven flow, electro-osmosis, or acceleration. One of the simplest approaches for producing flow in capillaries is to use capillary forces. Capillary-driven flow requires no peripheral equipment, and this concept is used for portable immunodiagnostic tests (31). One of the key parameters that determines whether the capture kinetics of an analyte is reaction-limited or transport-limited is the velocity of the analyte solution in the microchannel (32). At lower flow rates, analyte exploitation increases but the binding reaction becomes transport-limited, because lower volumes of analyte solution are available at the capture area per unit of time and thus fewer molecules can be captured per unit of time. In contrast to the transport-limited case, molecules in the sample volume are used more efficiently. The flow velocity of the analyte solution in the microchannel is probably the parameter that is the most practical to vary, using microfluidics with capillary pumps or forced evaporation.

The dynamic range of sensitive assays will mostly be limited saturation, whereby high analyte concentration leads to fast and complete coverage of all binding sites, which makes it impossible to distinguish between different elevated concentrations of analyte. Analyte dilution, which solves this problem for assays using microtiter plates, is not an ideal solution for a microfluidic-based immunoassay (32).

One of the long-term goals in the field of microfluidics is to create integrated, portable clinical diagnostic devices for home and bedside use, thereby eliminating time-consuming laboratory analysis procedures (see Central laboratory vs. point-of-care testing). This system is now available and is particularly used in emergency units.

Routine vs. specialty testing in the clinical laboratory

The transition of technologies applied in proteomics research, in particular protein arrays, to routine IVDs depends on test accuracy (precision and trueness). In addition, polyclonal antibodies used in the prototype assays will be replaced by monoclonal antibodies for commercial products. Thus, the microtiter plate (MTP) format used in phase II will have to be converted to an automated assay (Figure 1) for routine determination. This replacement will have to be verified by

extensive tests to show the equivalency of the two antibody classes. The automated assay system differs from the MTP format by a much faster result (few minutes vs. hours for MTP assays) (2).

In the clinical laboratory, protein determination is actually carried out using fully automated technologies such as turbidimetry, nephelometry and heterogeneous immunoassay systems based on magnetic beads. The throughput capacity is acceptable for all automates on the market, with theoretical rates of 800, 180 and 240 tests/h for Olympus AU640, Beckman Immage[®] 800, and Bayer Advia Centaur[®] systems, respectively. However, a majority of the techniques used for the measurement of soluble analytes involve ELISA-based technology. Although this methodology is well suited for single analyte analysis, it is more desirable to simultaneously measure multiple analytes in a relatively small sample (12–100 μL , according to the technique used). It is clear that sample size is a critical factor for the evaluation of multiple analytes, especially when monitoring disease states (30). Thus, multiplex assays for the detection of several proteins in a blood sample are very desirable.

As an alternative to planar microarrays, two multiplexed immunoassays are commercially available: microsphere-based assays and Protein Biochip Array Technology (PBAT) developed by Luminex and Randox, respectively. In the first system, miniaturized sites are placed on a three-dimensional surface, while a two-dimensional surface is involved in the second system. In the case of three-dimensional surfaces, each has a unique immobilized protein or capture agent, which is pooled into groups for parallel analysis. In the case of two-dimensional surfaces, multiple spots are arrayed, each containing a different immobilized protein or capture agent.

Microsphere-based assays Multiplexed assays using fluorescent microspheres have been developed and are making an impact in the laboratory. Three of the manufacturers of microsphere-based assays are Becton-Dickinson (Franklin, NJ, USA), Diasorin (Stillwater, MN, USA) and Luminex Corp. (Austin, TX, USA). We focus on the Luminex technology, including the Luminex 100 xMAP system (formerly called LabMAP, laboratory multiple analyte profiling). This method uses microspheres (commonly referred to as beads) as the solid support for a conventional immunosorbent assay with a green-fluorescent reporter dye. The capture antibody for each immunosorbent assay is coupled to one of 100 different microsphere bead sets (Figure 3). The system uses a liquid suspension array of 100 sets of 5.5- μm beads, each internally dyed with different ratios of two spectrally distinct fluorophores to assign it a unique spectral address. Each set of beads can be conjugated to a different capture molecule. The conjugated beads can then be mixed and incubated with samples in a microplate well to react with specific analytes. Capture molecules can include enzyme substrates, DNA, receptors, antigens, and antibodies. To detect and quantitate each captured analyte, a fluorescently

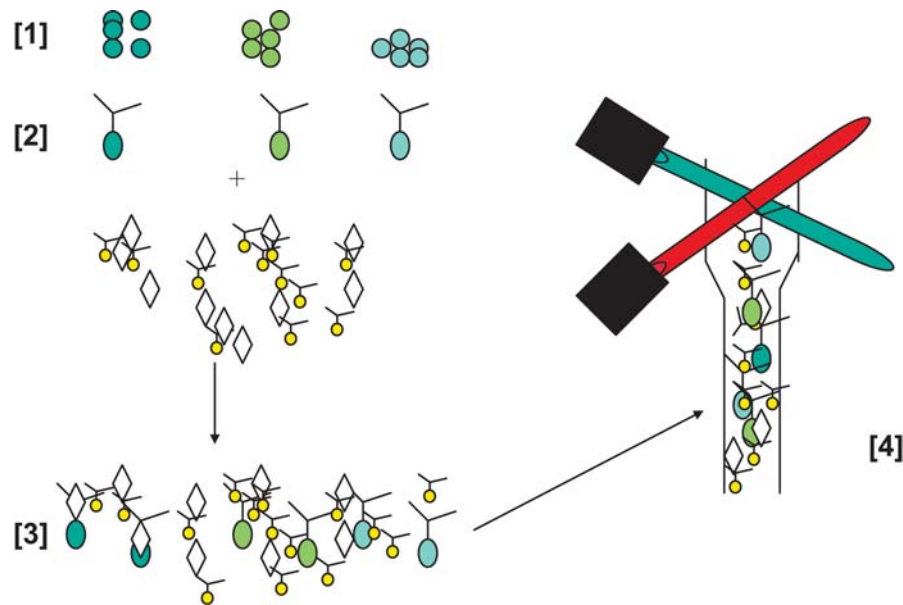


Figure 3 Microsphere-based assays. [1] The system uses a liquid suspension array of 100 sets of 5.5- μm beads, each internally dyed with different ratios of two spectrally distinct fluorophores to assign it a unique spectral address. [2] Each set of beads can be conjugated with a different capture molecule. [3] The capture molecule conjugated to a color-coded bead binds to a target analyte, followed by binding to a biotinylated detection antibody. [4] A red classification and a green reporter laser illuminate individual beads to identify each bead's spectral address and associated reporter signal.

labeled reporter molecule that specifically binds the analyte is added. Following incubation, the contents of each microplate well are drawn into the array reader, and precision fluidics align the beads in single file through a flow cell, where two lasers excite the beads individually. The red classification laser excites the dyes in each bead, identifying its spectral address. The green reporter laser excites the reporter molecule associated with the bead, which allows quantification of the captured analyte. High-speed digital signal processors and software record the fluorescent signals simultaneously for each bead, translating the signals into data for each bead-based assay.

The principal advantages are its multiplexing capability, its flexibility, its precision (33–35), cost and the time taken to perform the assays (theoretically 3 h to obtain data on 17 cytokines for 80 samples). A sample volume of only 12 μL , which is generally not sufficient for analysis of a single protein by ELISA, is required for the assay of 17 cytokines. This advantage increases with each additional parameter analyzed. Minimal training is required to operate the Luminex 100. The versatility of the technology also allows protein, RNA, or DNA testing to be performed on the same system, while its throughput capabilities can be applied to any size laboratory for clinicians and researchers.

A number of companies have licensed the Luminex technology and are marketing or are planning to market kits for research applications. By creating partnerships with research, drug discovery companies, such as Bio-Rad Life Science, Linco Research and Qiagen, and clinical diagnostic companies, such as BMD and Immune Tech, many applications and options may be made available. A wide range of applications have been developed, including allergy testing, autoimmune diseases, cancer markers, cardiac markers,

cytokine, endocrine and metabolic markers, infectious diseases and kinase/phosphorylated protein. We may choose to build custom assays from the Luminex carboxylated xMAP microspheres, or to take advantage of the many kits available.

For some applications, the lower limits of the standard curves will be the limiting factor of the assay. For example, in a study by de Jager et al. (34), the lowest concentration that could be detected in the linear part of a standard curve for interleukin 4 (IL-4) was approximately 40 pg/mL, whereas a sensitive ELISA for IL-4 would have a lower detection limit. A recent study (35) reported poor correlation with conventional ELISA methods and low detectability for multiplexed analysis of biomarkers related to obesity and the metabolic syndrome. In addition, data acquisition with this system becomes more complex when a large number of samples are measured with a multiplex assay (34). The multiplex assay involves mixing a large number of different antibodies in a single reaction. These antibodies could bind non-specifically to any other component within the assay and serve as antigens for other immunoglobulins, causing false-positive values or blocking of the readout (36). Thus, when serum is used as the matrix, it has to be carefully monitored for blocking substances such as heterophilic antibodies and for the presence of cytokines, even when used at high dilution. De Jager et al. (34) encountered this problem for an IL-13 standard curve, which was blocked completely by both ELISA and the Bio-plex system when a cell culture matrix was used. HPE buffer resulted in a good readout. This effect was overcome by switching to another batch of human serum that was tested for such a blocking event and did not contain any detectable cytokines. Antibody specificity is essentially limited by the quality of the

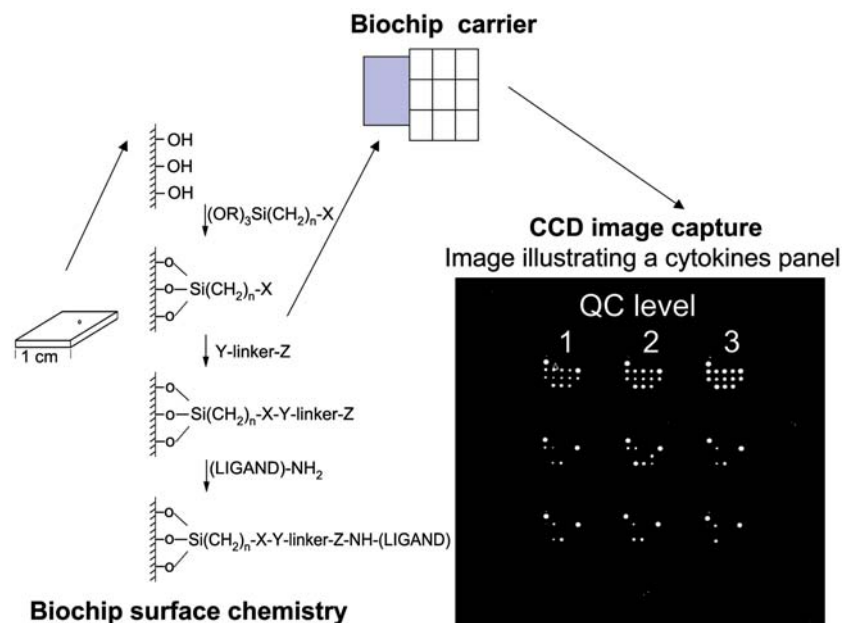


Figure 4 PBAT developed by Randox. The biochip is a 9-mm² solid substrate with multiple specific ligands (antibodies or antigens) attached at pre-defined sites on the surface. Biochips are presented in a 3×3 carrier. The degree of binding is determined using a chemiluminescence light source and quantified using a charge-coupled camera (CCD) and imaging system. The example image shows a cytokine panel (12 cytokines) with quality control levels 1, 2 and 3, and samples.

antibodies used in assays and it is likely that significant emphasis will have to be placed on the development of better antibodies. For the immunologist's point of view, the limitation of this technique is the availability of matched antibody pairs that do not cross-react in the system. In addition, the xMAP[®] technology, with an already extensive list of applications and partners, does not offer complete automation.

Protein Biochip Array Technology The latest technological innovation by Randox Laboratories (Crumlin, UK) is multi-analyte biochip array technology, including PBAT. Known as Evidence[®] and Evidence Investigator[™], the systems have been developed to allow simultaneous measurement of up to 25 analytes with a single drop of patient sample on a biochip. The biochip is a 9-mm² solid substrate with multiple specific ligands (antibodies or antigens) attached at pre-defined sites on the surface. Silanation was the most suitable method to activate the surface of the biochip and allow attachment of the ligand. The silane reacts with the hydroxyl group on the biochip surface. A hetero-bifunctional linker is introduced, which reacts with the silanated surface and provides the reactive group. This group is then capable of reacting with the NH₂ groups of a ligand, which in most cases will be an antibody, but could also be a peptide or a nucleotide. Binding of the ligand to the surface of the chip is not the only problem, however. It is also essential that the antibody remains active once attached (Figure 4).

This technology utilizes the principles of immunology, supporting competitive, sandwich and antibody capture immunoassay formats. The chemiluminescence signals are simultaneously measured for the

full array of tests on each chip. Biochips are presented in a 3×3 carrier, which facilitates transport within the analyzer.

Evidence[®] is a fully automated clinical profiler designed for high-throughput laboratories (37, 38). The system boasts an output capacity in excess of 3500 tests/h. After addition of a patient sample to the biochip, analytes present in the sample bind to specific ligands. The degree of binding is determined using a chemiluminescence light source and quantified using a charge-coupled camera (CCD) and imaging system. Dedicated software performs all system operations from on-board maintenance to calibration validation and patient sample profiling for a variety of tests. The most important advantage of this biochip system is that it can be fully automated to provide high throughput, leading to 3600 tests/h, acceptable performance (37, 38) and ease of use. In future, the capacity of tests per biochip may be dramatically increased to 100.

The Evidence Investigator[™] is a bench-top biochip system comprising a super-cooled CCD camera and unique image-processing software, utilizing the same biochip array technology as the Evidence[®] system. This system offers multiplexing: one patient sample will provide multiple results. The state-of-the-art technology is user friendly and offers a respectable throughput for a wide range of protein assays (e.g., 3 h to obtain data on 12 cytokines for 42 samples), clinical trials and research applications. This system utilizes the same biochip as the Evidence[®] system, so all panels available can be used. In addition, Evidence Investigator[™] is more compact than the Evidence[®] system, so is more suitable for the clinical laboratory.

The range of biochips available includes drugs of abuse (DoA), cardiac markers, fertility hormones, thy-

roid hormones, cytokines and growth factors, tumor monitoring and prostate screening. Many more protein and DNA biochips are in development, which will be available in the future for clinical research and clinical trials. These include allergen arrays, adhesion molecule arrays, growth-promoter residue arrays, anemia arrays and antibiotic drug residue arrays.

Furthermore, Randox has a very large-scale project ongoing to develop a vast range of tests for novel markets in a variety of disease conditions, including breast cancer, ovarian cancer, colorectal cancer, esophageal cancer, cardiovascular disease, stroke and traumatic brain injury. PBAT can be customized for use in pre-clinical and clinical tests on demand.

A few studies have reported data concerning PBAT developed by Randox (37–39). First, precision data (37, 38) and correlation studies (38) for the cytokine panel were reported. Calibration curves for all markers in each of the test panels (10 cytokines) cover a large dynamic range, but it is clear that the low-value calibrator is lower than typical ELISA detection limits. As a consequence, poor correlation with ELISA methods is expected for low values. In addition, the functional sensitivity is near the limit of detection of the assay (37). Second, PBAT for a cardiac panel was evaluated for the diagnosis of acute coronary syndrome (39). For all the markers included in the panel, imprecision studies revealed good results, with total CVs of 5.9–9.7%, except for troponin I in a serum pool at a low level (17% CV). Over the full concentration range, global correlation between the reference method (Dimension RxL, Dade Behring, Paris, La défense, France) and the Evidence Investigator™ panel was good, but positive significant and absolute bias was evident at low concentrations. The manual washing procedures for the biochip surface might be the cause of higher background noise not completely removed by software processing of the image. Results of performance studies and the high background should be improved for the Evidence® system because automation is complete.

Central laboratory vs. point-of-care testing

This section reports other applications in the field of microfluidics. By reducing the amount of fluid handled to the nano- or picoliter range, microfluidic systems miniaturize chemical and physical analysis onto a chip size. Such systems are particularly adapted for high-throughput protein-based diagnostics in healthcare applications. The availability of improved therapeutic interventions for acute coronary syndrome, for example, makes rapid diagnosis and disposition of patients with these conditions essential. POC testing is one alternative for providing fast results in emergency departments.

In 1999, Biosite commercialized Microfluidics Chip-Based Immunoassays and manufactured the Triage protein chip, which is a POC system. The first chip was the Triage® Cardiac panel, which uses 10 antibodies arrayed in six zones. The test identifies three cardiac protein markers (myoglobin, troponin I and creatine kinase) that are released in the blood and

increase during an acute myocardial infarction. Triage protein chip immunoassays are performed on a microfluidic plastic chip, and the results are achieved in 15 min with picomolar sensitivities. The microfluidic flow is controlled passively in the protein chip by the surface architecture and surface hydrophobicity in the microcapillaries. The immunoassays use high-affinity antibodies and a near-infrared fluorescent label, which is read by a portable, battery-powered fluorimeter. As previously described, Biosite's protein array is comprised of two components: the protein array device, and the Triage protein chip, which we next describe.

Assay principle When a sample of blood is added to the protein array, a fibrous filter contained within the array separates red blood cells from plasma. The plasma then exits the filter and flows into a region of higher capillarity, called the sample reaction barrier.

The plasma then enters a reaction chamber of lower capillarity, which contains reagents in a dry form. The plasma reconstitutes the reagents to form a reaction mixture. The reagents include antibodies bound to fluorescence energy transfer particles (FETL). A time gate delays the flow of plasma out of the reaction chamber to define an incubation time. This time gate is composed of hydrophobic surfaces that prevent fluid flow. These surfaces are converted into hydrophilic surfaces by the proteins in the sample, allowing the reaction mixture to flow into the diagnostic lane.

The diagnostic lane is a capillary channel containing discrete zones of immobilized antibodies to the analytes. Any analytes bound to FETL from the reaction mixture will bind to the immobilized antibodies as the reaction mixture moves through the diagnostic lane. Excess plasma from the blood filter washes unbound FETL from the diagnostic lane. The fluorescence in each discrete zone is measured by a handheld fluorimeter called the Triage MeterPlus. When inserted into the Triage MeterPlus, the protein array device is scanned by a laser diode. The FETL fluorescence emission is detected by a photodiode in a high-gain circuit. The fluorescence intensity is integrated over each discrete zone to give a quantitative measurement of the analyte concentrations (Figure 5).

Triage platforms Biosite technologies have been used to develop two novel, rapid testing platforms: the Triage Panel and the Triage MeterPlus. Both platforms deliver highly accurate results within 15 min.

The Triage Panel, a qualitative platform, allows rapid screening of multiple analytes in a small, handheld, single-use device. It contains a visual "yes/no" display allowing simultaneous diagnostics for any combination of analytes and control standards. The Triage Panel products include Triage® Drugs of Abuse Panel (used for the detection of nine classes of abused drugs), Triage® *C. difficile* Panel (used for the detection of *Clostridium difficile* common antigen and toxin A in human fecal specimens), and Triage® Parasite Panel (used for the detection of antigens specific for

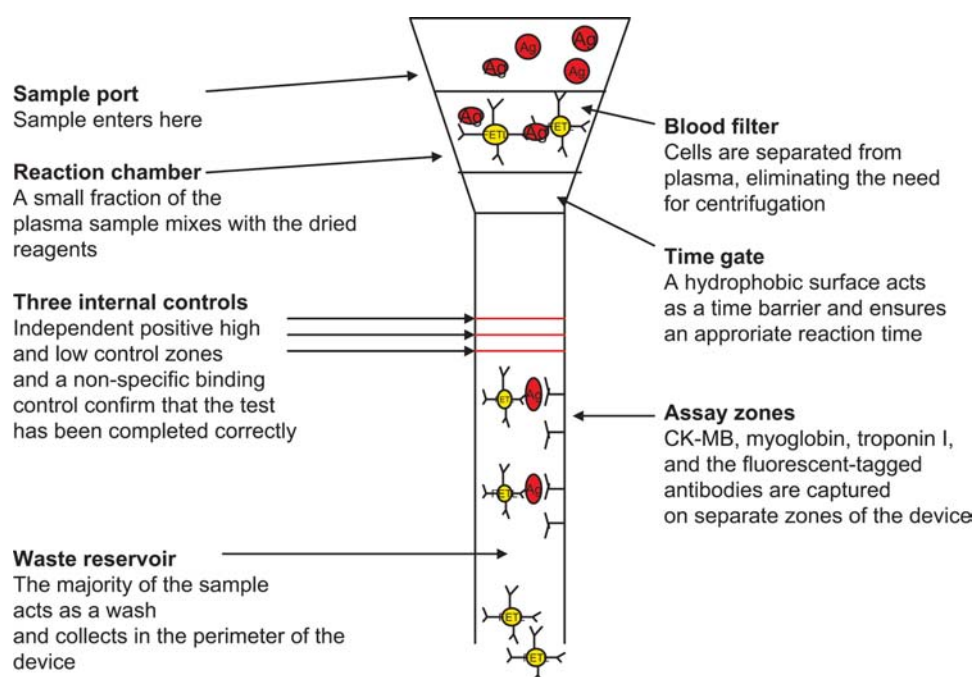


Figure 5 Assay principle of the microfluidic Triage MeterPlus (Biosite, San Diego, CA, USA). Ag, antigen (e.g., cTnI, CK-MB, myoglobin); FETL, fluorescence energy transfer particles.

Giardia lamblia, *Entamoeba histolytica/dispar*, and *Cryptosporidium parvum*).

The Triage MeterPlus platform uses a one-step process to provide rapid, quantitative or qualitative results for immunoassays using urine, whole blood, or plasma. The Triage MeterPlus products include Triage® Cardiac Panel (quantitative measurement of CK-MB, myoglobin, and troponin I), Triage® BNP Test (brain natriuretic peptide levels), Triage® Profiler Shortness of Breath Panel (measurement of troponin I, CK-MB, myoglobin, BNP and D-dimer), and Triage® TOX Drug Screen (determination of nine drugs).

Biosite is focusing on the detection of protein POC immunoassays to aid in the diagnosis and assessment of patient care. A description follows of some of the important product features and potential benefits to the POC user. Results of all markers are available simultaneously within approximately 15 min after sample addition. Simple one-step testing is involved, since the system allows the use of whole blood samples. Built-in quality control features and lot-specific calibration ensure accuracy and high quality results. The system needs low maintenance and is designed for compatibility with busy emergency departments and physician practices (31). In addition, the system is compact and portable. Several studies have reported that this system has the ability to support accelerated care pathways and to decrease the length of stay in the emergency department (31, 40).

Triage platforms have the same advantages and inconveniences as microfluidic technology. However, because it is a portable instrument used by non-medical personnel, it is important to report practical considerations. The external quality control (QC) solutions provided consist of a Cardiac Calibration Verification Control Set at three concentrations in a

plasma pool and a Cardiac Control Set at two concentrations in artificial matrix solutions. The QC solutions should be stored at -20°C or for 15 days at room temperature, which may be problematic. The acceptability of QC values is large, at three standard deviations (± 3 SD). If the two internal controls fail, the test must be repeated. Furthermore, the meter has the following features: QC requirements programmed into the instrument, lock-outs for unauthorized personnel, and a laboratory information system interface. If a problem affects sample transport, false negative results may be observed. Dilution is not advised and not adapted for this instrument.

Selection of a POC testing system goes beyond the need to provide rapid, accurate, and reliable results.

Conclusion

The development of lab-on-chip systems will have to evolve with changes in proteomics to remain relevant. This technology must prove useful and practical in obtaining objective data. Biochip technology is in an expansive phase that is clearly limited at the technology level. Recent advances in biochip assays combine the power of miniaturization, microfluidics, micro- to nanoparticles, and quantification to provide total automation, the highest sensitivity, multiplexed assays, lower reagent costs and a high degree of parallel data collection. Many efforts have also been undertaken in the field to launch products for the diagnostic market with excellent precision, sensitivity and reliability. Screening, diagnosis and monitoring of diseases can be revolutionized by biochip technology. The multiparallel possibilities of protein biochip applications have the potential not only to allow the

optimization of pre-clinical, toxicological and clinical studies through better selection and stratification of subjects, but also to affect how diagnostics are used in drug development. Multiplexing increases the amount of information obtained from a single sample and enables researchers to examine profiling. This is crucial for many parameters where proteins such as cytokines, function in concert. Multiplexing can increase the sensitivity and specificity of biomarkers by combining markers that have only limited predictive power as singlets but high predictive power in panels (41). A number of applications are just beginning to be explored. The power of biochip assays offers great promise for POC clinical testing and monitoring of many important analytes.

Proteomics has been an important area of research for the past two decades. Advances in technology have led to a rapid increase in applications to a wide range of samples and use as an extension of the clinical laboratory. The biochip represents a new technology platform that is available now and is expected to have a significant impact on clinical diagnostics and medical research in the near future.

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