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xMAP Suspension Bead Array Technology

Challenges in Comparing Results to Those of Traditional Methods for Selected Applications

The Bead Array Strategy

Traditional biochemical methods often detect or quantitate proteins individually. Unfortunately, individual analysis prevents researchers from elucidating complex biochemical pathways and processes. Today, protein arrays enable researchers to simultaneously measure multiple analytes. Luminex xMAP technology was the first — and continues to be the most successful — bead array strategy designed for medium-density, medium-throughput array applications. The technology uses spectrally addressable microspheres as array elements to enable the simultaneous analysis of up to 100 analytes in only a few microliters of sample (see Figure 1). The large surface area of suspension arrays and the high concentration of reactants on the surface of the beads improve binding kinetics, reducing assay time and increasing assay sensitivity (de Jager et al. 2003). The Bio-Plex™ suspension array system integrates this powerful bead array technology with instrument control and data analysis software, system validation and calibration tools, multiplex immunoassays, and all the supplies needed to perform xMAP-based assays in a 96-well microplate format.

This article highlights some of the validation and correlation issues involved when applying xMAP technology to specific applications. We will look at two popular cell-signaling applications: cytokine testing and phosphoprotein testing. These applications leverage the strengths of xMAP suspension array technology — analysis of multiple proteins in complex biomolecular processes simultaneously. This technology also has potential utility in biomarker quantitation. The bead array strategy for testing allows the end user to mix and match targets rather than committing to a specific set of analytes (Figure 2). Furthermore, analysis takes place in a liquid medium, where proteins can be analyzed in their native or near-native conformation, rather than on the solid medium of a chip (see sidebar on next page).

Assay Data Categories

Before we discuss applications of a given method, it may be useful to categorize assays or applications by whether they are quantitative or nonquantitative (see Table). By breaking assay applications into these categories, we can gain insight into expectations and thus their performance. One way of looking at this is to sort assays into four distinct categories: definitive quantitative, relative quantitative, quasi-quantitative, and qualitative (Bowsher and Smith 2002).

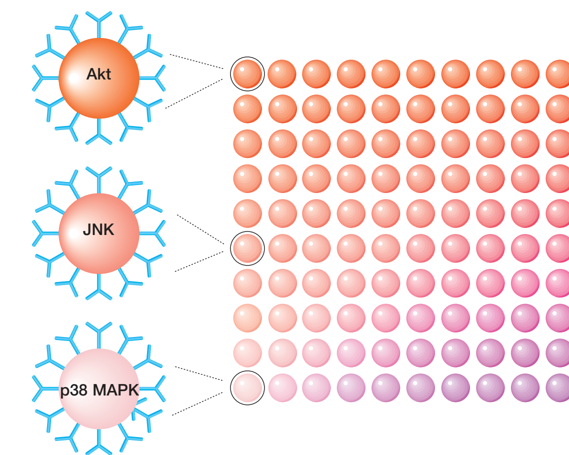

Fig. 1. Multiplexing using xMAP technology. xMAP technology utilizes 100 bead colors. The dyed 5.6 µm beads are automatically categorized into bead regions. By coupling the capture antibody to the different bead regions, xMAP technology allows simultaneous detection of each capture antibody.

Table. Comparison of the statistics and studies that can be performed on relative quantitation assays vs. quasi-quantitation assays.

	Relative Quantitation (Example: Cytokines)	Quasi-Quantitation (Example: Phosphoproteins)
Accuracy	Spike recovery	Not applicable
Precision	Replicate testing	Replicate testing
Sensitivity	LOD or LLOQ	LOD
Specificity	Cross-reactivity studies	Cross-reactivity studies
Linearity	Ratio of observed to expected values for diluted sample	Not applicable; however, the dose response of a diluted sample may suggest linearity
Matrix effects	Dilution studies	Dilution studies
Recombinant standard	Test vs. native protein	Not applicable

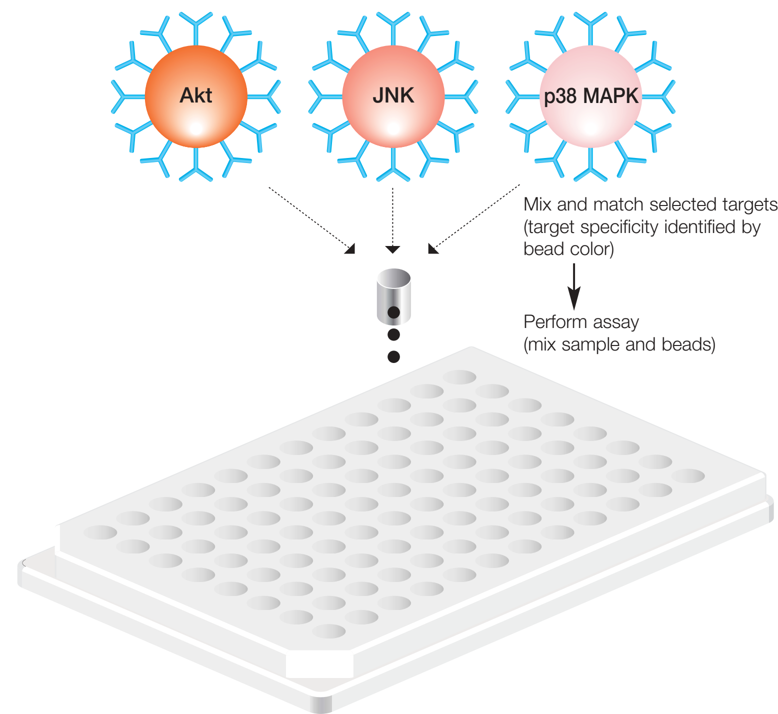

Fig. 2. Bead array strategy. Based on a mix-and-match approach, 5.6 µm coupled beads in suspension allow mixing of appropriately coupled regions in a single well. This enables multiplexing of up to 100 analytes simultaneously.

The ideal situation for the user is a definitive quantitative assay — meaning that there is a defined, endogenous reference standard available. While ideal, this usually is not the case for most novel markers, particularly in life science research. A good example is cytokines. While cytokines have been quantitated for years by ELISA, few endogenous, purified reference materials exist. Therefore, cytokine assays today fall into the relative quantitative category.

The quasi-quantitative result is defined by having a measurement that is characteristic, but that characteristic cannot be readily compared. Activity assays fall into this category. For example, assaying kinases (measuring phosphorylation) is possible by western blot, ELISA, and bead-based assays. Western blots will show lighter and darker bands, ELISAs will read a higher or lower optical density (OD), and bead-based assays will read a higher or lower median fluorescence intensity (MFI). But what does it mean if we want to compare results across methods? Unfortunately, the ability to generate a number on a scanner or plate reader can mislead us into grasping for quantitation-based data.

The last category, qualitative data, is generally a positive or negative type of result. For the purposes of this article, we will not be discussing qualitative results.

Once we've defined the parameters for evaluating methods, we can look at the challenges that different categories of assay data present.

Protein Bead Arrays — A Compelling Approach

Oligonucleotide and cDNA arrays helped revolutionize genomic research, in part due to the tremendous amount of information available from a single experiment. Similarly, protein arrays are making large-scale proteomics more feasible, and coupled with powerful computational methods are facilitating a more system-wide approach to biological research. Protein microarrays (chips) are a logical extension of the DNA array, but while microarrays monitor gene expression levels in a particular organism, only protein microarrays can provide similarly comprehensive information about the activity and function of those gene products.

While traditional biochemical methods have been invaluable in elucidating protein function on an individual basis, such methods cannot reasonably be used to evaluate an entire cellular proteome or tease apart a complex network of biomolecular processes. Deciphering even a single protein's function in a complex biochemical pathway typically requires years of intense study using traditional methods. It is here that protein arrays may have the greatest impact, for they provide both the high-density format and the multiplexing capability that are required for such global analysis.

Current assay tools, such as 2-D PAGE, mass spectrometry, enzyme-linked immunosorbent assay (ELISA), and similar techniques are proven methods for expression proteomics and drug development. While effective, these methods can be cumbersome, suffering from low throughput, low data density, or both. Protein arrays using microbeads combine the simplicity of ELISAs with the multiplexing ability of microarrays. Protein bead arrays, like their nucleic acid counterparts, are poised to drive the study of protein systems by offering both higher sample throughput and a higher density of results for a given sample (Willis et al. 2003).

Protein arrays utilizing microbeads enable parallel analysis of multiple proteins, often requiring much smaller sample volume for a single experiment compared to an ELISA or western blot. In addition, bead arrays are typically amenable to a range of sample matrices, enabling analysis of many types of biological samples, including blood filtrates (Hutchinson et al. 2001), culture supernatants (Rutella et al. 2002), tissue homogenates (Hulse et al. 2004), and cell lysates. Because of the variety of samples that can be analyzed using protein arrays, a correspondingly large number of applications can benefit from the use of protein bead arrays.

Quantitation of proteins in biological samples is one application that is particularly amenable to multiplex analysis, leading to protein expression profiling or a molecular signature approach to sample analysis. However, protein bead arrays are not limited to expression analysis: Preferential enzyme target recognition, protein regulation and signaling activation (e.g., phosphorylation), binding site recognition and competition, and other functional assays have been developed, and several other assay formats are possible using the same core technology. Thus, like DNA microarrays, protein bead arrays provide a "high content" solution to protein analysis.

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Protein Profiling — Cytokines

Cytokines are important extracellular signaling proteins, mediating a wide range of physiological responses, including immunity, inflammation, and hematopoiesis (Jenmalm et al. 2003). They are also associated with a spectrum of diseases ranging from tumor growth to infections to Parkinson's disease. While cytokines do exist intracellularly, most research involves measuring extracellular concentrations — generally in cell culture supernatants or in serum.

Cytokines are typically measured by immunoassay, most commonly using a sandwich assay format. The cytokines of interest are captured by an antibody that is specific for the target and detected by a secondary antibody directed against a different epitope. Presence of the target cytokine is read optically, and the data are displayed digitally (as OD when obtained by ELISA or MFI when obtained by bead-based assays using xMAP technology). The data obtained for several known concentrations generate a curve. Unknown sample concentrations are then determined by interpolation based on the standard curve.

Issues That Cloud Relative Quantitation of Cytokines

Lack of Endogenous Standard Material

No endogenous standard exists for cytokines today. The only alternative to a true reference standard in cytokine testing is the National Institute for Biological Standards and Control (NIBSC) standards. However, the NIBSC standards do not qualify as true reference materials because many are recombinant proteins that may differ from natively expressed proteins found in a biological sample. Because they are recombinant, the possibility of posttranslational modification — such as glycosylation, which may alter an assay's response — cannot be ignored (Wadhwa et al. 1999). Making matters even cloudier, the NIBSC does not assay its standards for concentration (for example, pg/ml values reported by immunoassay methods), but rather reports in international units (IU) based on bioassay activity. Thus, many assay manufacturers provide IU conversion factors that link soluble concentration to biological activity.

When manufacturers and researchers create cytokine assays, they generally use recombinant material readily available to them — meaning each may be slightly different (Ledur et al. 1995, de Kossodo et al. 1995). This is not to say that the results of these assays are meaningless. On the contrary, there is generally a reasonable correlation between different assays. But to make sure there is a reasonable correlation, the assay curve must be tested against native material (for example, lipopolysaccharide-stimulated peripheral blood mononuclear cell supernatant).

Antibody Variation

Another key component of an immunoassay is the reagent used for analyte capture and detection. Antibodies are perhaps the most widely used reagents in protein microarrays, because they can be made highly specific for their targets and are often able to differentiate subtle protein modifications such as phosphorylation. The sandwich immunoassay format is often used, making use of two antibodies that simultaneously bind the same target (Figure 3). Sandwich assays offer the advantage of very high specificity, given that both antibodies must recognize a common target for successful measurement. Nevertheless, the use of antibodies can complicate the direct correlation of results between platforms, as each platform may use antibodies whose epitopes may differ between sets of capture and detection reagents. While recognition by one antibody for an epitope may be masked or obscured through biomolecular interactions involving soluble targets, another antibody's target epitope may be free from such interference, thereby recognizing both complexed and uncomplexed forms of the target molecule. This may or may not be desirable, depending on the context of the experiment and the specific molecular target. Furthermore, recognition may differ between native and recombinant proteins.

To reduce the possibility of variation, monoclonal antibodies are the first choice for an assay because variability in the affinity for the epitope is minimal. Unfortunately, specific monoclonal antibodies are not always available for novel targets, and the high degree of antibody characterization that is desirable is difficult and often complex (Wadhwa et al. 1999). This complexity is further compounded when developing a multiplex kit — each antibody, either detection or capture, must be checked for cross-reactivity against all other antibody pairs that are to be combined. Generally speaking, high-multiplex assays require increasingly cumbersome and difficult validation compared to their low-multiplex counterparts.

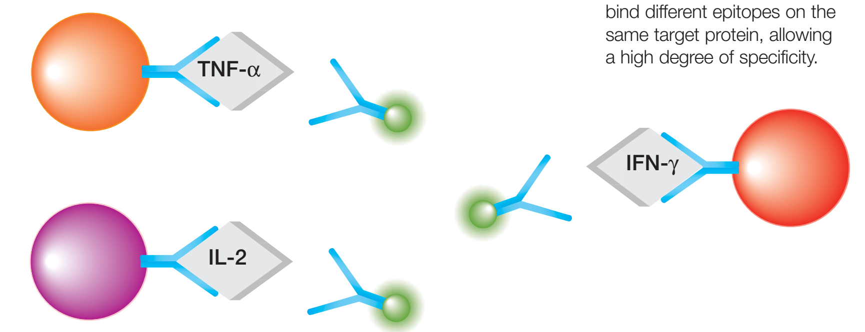

Fig. 3. Multiplex sandwich immunoassay. Two antibodies simultaneously bind different epitopes on the same target protein, allowing a high degree of specificity.

Extracellular Samples

Potential effects of the matrix (for example, serum or plasma) can be addressed by making a dilution series of spiked material in the matrix of interest (de Kossodo et al. 1995). It is always possible that in some samples, high levels of interfering factors may cause unusual results. How do we rule this out? It is our experience that running dilutions of the (unexpectedly) high-concentration sample will lead to the correct answer. Upon dilution, the interfering substance(s) become dilute enough that nonspecific binding drastically drops, leading to a nonlinear dilution series for the sample of interest. If it is in fact a nonlinear dilution series, it can be inferred that an interfering factor is likely to have been present in the sample.

For clinical study support, characterizing normal and test populations of patients is important (Findlay et al. 2000). We tested the sera of 50 apparently normal human samples using the Bio-Plex human cytokine 17-plex panel and observed a range of values (Figure 4).

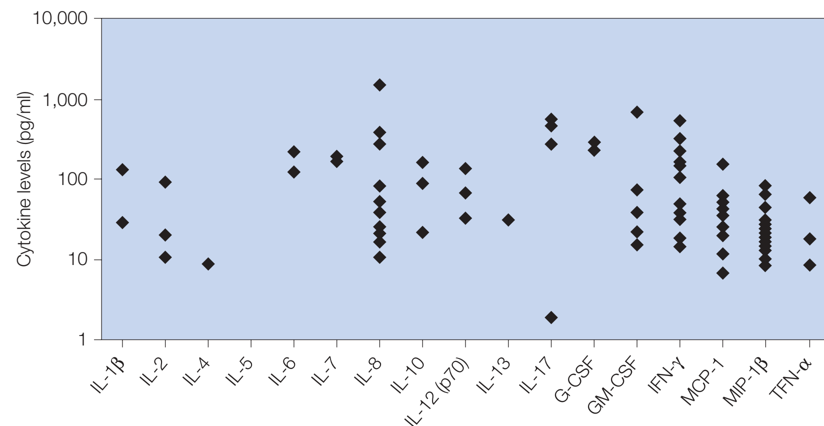

Fig. 4. Cytokine expression profile. Sera from 50 normal human samples were tested using the Bio-Plex human cytokine 17-plex panel. Results for each analyte are shown. For analytes that display fewer than 50 points, the remaining values were below the 2 pg/ml limit of detection.

Monitoring Signal Transduction — Phosphoproteins

Bead array phosphoprotein assays allow the simultaneous analysis of the phosphorylation state of several target proteins within a single cell lysate sample. The principle of phosphoprotein assays using xMAP internally labeled beads is similar to that of a capture sandwich immunoassay. An antibody directed against the desired phosphoprotein target is covalently coupled to 5.6 µm fluorescently dyed beads. The coupled beads are allowed to react with a sample containing an unknown amount of phosphoprotein target. After a series of washes to remove unbound protein, a biotinylated detection antibody specific to a different epitope on the target protein is added to the reaction. The result is the formation of a sandwich of antibodies around only phosphorylated target protein. The reaction mixture is detected by the addition of streptavidin-phycoerythrin, in which the streptavidin binds to the biotinylated detection antibodies.

Western blot analysis is the most common method of testing for phosphoproteins today. In short, the western blot method consists of denaturation of proteins (heated in buffer containing SDS and 2-mercaptoethanol), gel separation, and transfer onto nitrocellulose membrane, followed by specific antibody probing. While western blot analysis has proven to be a commonly used method of testing phosphorylated proteins, it tends to be time-consuming and is subject to variability between runs. Phosphoprotein testing by western blotting generally involves testing a control sample that is unstimulated or normal against a test sample that is stimulated or treated with an inhibitor. A significant difference in signal (as measured by a scanner) is often used to compare the control against the stimulated sample (for example, signal is 3-fold higher in the stimulated sample vs. the control).

Issues That Cloud Quasi-Quantitation of Phosphoproteins

Lack of Phosphorylated Standard Material

It is important to make the distinction in quantitation categories at this point. As opposed to the relative quantitation of cytokines, phosphoprotein testing is quasi-quantitative. No phosphoprotein standard is used in western blot analysis, which is why a control sample is run against a test sample. So how do we correlate results from a western blot and an immunoassay method? One common method of analysis for western blots is a ratiometric approach, resulting in a "fold-change" of a test sample over a control. However, there are potential dangers involved in applying this same analytical methodology to other platforms, in part due to the lack of standard material for calibration purposes. Fold-change, as determined by most assay platforms, does not necessarily correlate directly with the absolute magnitude of protein expression or modification. By necessity, a fold-change calculation requires a linear response over the test sample range. Furthermore, fold-change will often vary between platforms because the linear region of the dynamic range is highly platform dependent, even showing significant variation between different methods of staining or visualization. Results from western blot experiments are typically obtained by image analysis and densitometry, and that digitized, "quantitative" information then forms the basis for further numeric manipulation and analysis.

Finally, numerical analysis must be comparable. For example, to minimize variation between western blotting and Bio-Plex assay, it is recommended to use the same lysate (Han et al. 2003) and labeled detection antibodies. Ratiometric results (fold-change) should likewise be calculated in a similar manner, including background subtraction, signal averaging, etc.

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Antibody Variation

As mentioned before, any immunoassay is subject to differences between antibodies used. A major difference between a western blot and a bead assay (or any sandwich assay) is the number of antibodies needed; a western blot requires only a single detection antibody, whereas bead arrays require an antibody pair to complete the "sandwich". On one hand, this may lend better specificity and reduced background to the bead-and-sandwich approach because it means that two highly specific antibodies, directed at different epitopes, are needed to run a bead assay. On the other hand, the sandwich method requires two highly specialized reagents, potentially complicating development.

A major issue that plagues phosphoprotein assay development in any platform is the lack of phospho-specific antibodies. The limited availability of high-specificity, high-affinity antibodies is compounded by the fact that the antibodies must be further modified by immobilization onto beads or labeled with fluorophores without damaging their functionality.

Intracellular Samples

In contrast to cytokines, which are generally tested extracellularly (that is, in serum, plasma, etc.), phosphoproteins are intracellular proteins and thus require cell lysis for accurate measurement. As such, the separation of phosphoproteins of interest from their intracellular complexes becomes a significant consideration in an assay system. This task is further complicated by the many cell and tissue types, each of which may respond differently to the wide variety of available cell lysis methods. Commercial phosphoprotein blotting kits generally include cell lysates that serve as control samples to demonstrate antibody specificity against the target of interest. Lysates provided with Bio-Plex phosphoprotein and total target assays serve the same purpose.

As more assays are introduced, the threat of cross-reactivity becomes an issue. There will likely come a point where it will not be possible to introduce new assays into a high-multiplex assay without encountering antibody cross-reactivity with other target proteins. Therefore, it may be necessary to divide samples over two or more assays in order to accomplish a high-multiplex analysis involving many targets.

Conclusion

All methodologies have strengths and weaknesses that are often judged based on a correlation of results to traditional methods. Multiplex bead-based assays are no different — they are compared to existing immunoassay methods (e.g., ELISA, western blotting, etc.). Many of the differences among immunoassay methods arise from the fact that immunoassays depend on the antibodies and proteins used (Findlay et al. 2000). With increasing acceptance of the bead-based xMAP technology, issues regarding sensitivity, specificity, and calibration will be further clarified by each new validation study. The growing number of studies in the literature that employ bead-based xMAP technology suggests that this strategy for protein testing is here to stay.

Proteomic Applications: Chip vs. Bead Array

As applied in proteomics research, suspension bead arrays rival the advantages of the most promising protein chips. Synthesis of suspension arrays takes advantage of established and readily available protein capture molecules, such as specific antibodies and binding proteins. Multiplex protein assay protocols are straightforward, often similar to classic immunoassays. The relative ease of assay generation and simplicity of protocols make suspension bead arrays a valuable research tool for protein expression profiling, detection of protein posttranslational modifications, and screening for protein functions and interactions.

While chips may have an advantage in content per sample, bead arrays clearly have an advantage in flexibility. Protein chips consist of a solid medium (e.g., glass slide) that is "spotted" to capture the analytes of interest. The predetermined number of analytes on the medium cannot be modified. In contrast, bead arrays consist of different populations of beads directed against different targets. This allows the user to mix and match beads directed against specific targets (Figure 3).

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