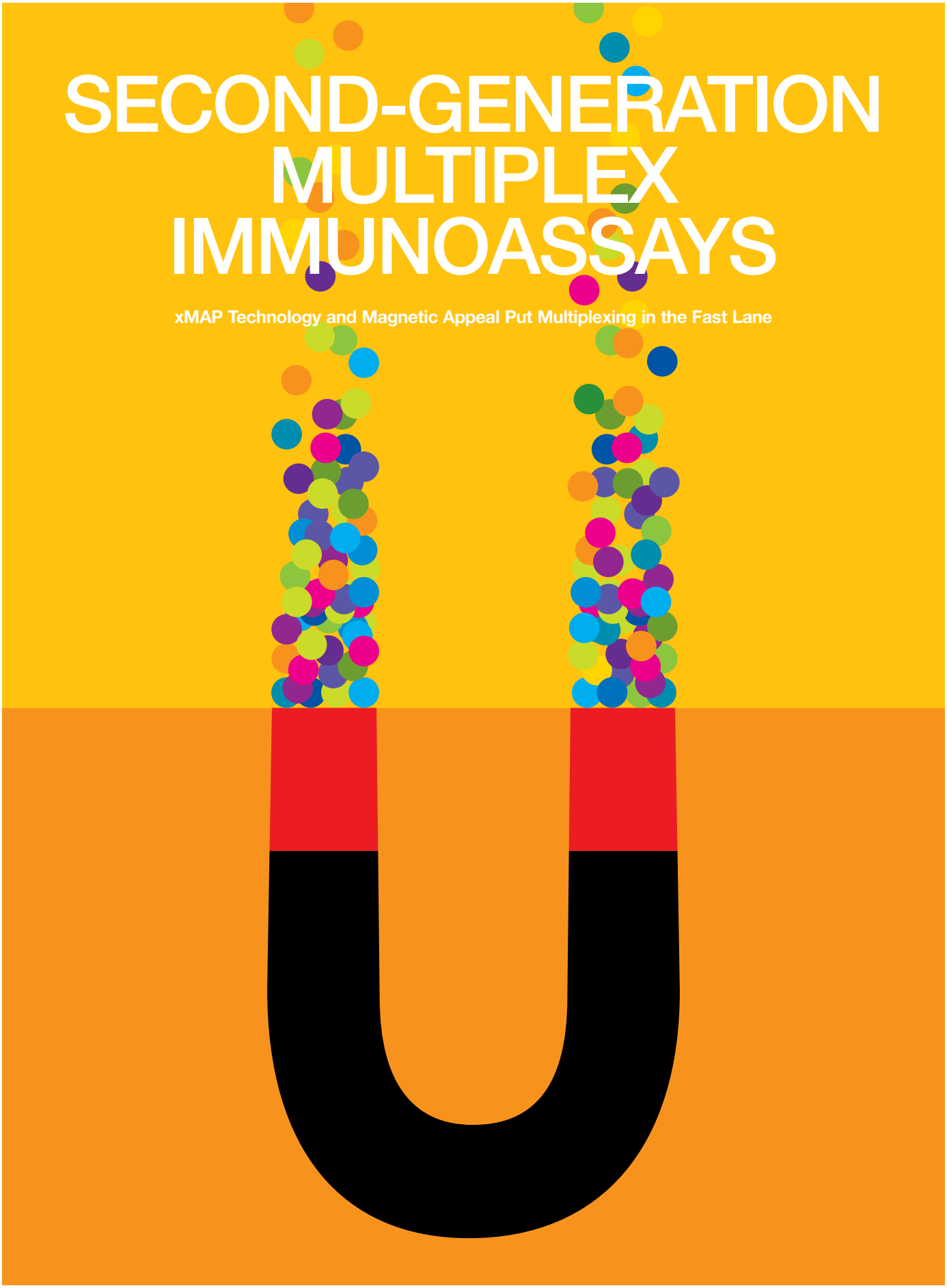
SECOND-GENERATION MULTIPLEX IMMUNOASSAYS

xMAP Technology and Magnetic Appeal Put Multiplexing in the Fast Lane



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For almost 50 years, immunoassays have allowed sensitive and highly specific detection of analytes of interest in biological samples for use both in life science research and clinical diagnostics. Immunoassays provide information to researchers on the roles proteins and other biomolecules play in a myriad of biological processes, thereby providing insight to clinicians on the identification and assessment of disease progression.

Early Immunoassays

The first immunoassay was developed by Yalow and Berson (1960), who received the Nobel Prize for their efforts to measure insulin levels. These initial assays used radiolabels for detection. The radioimmunoassay (RIA) would remain the standard for detection of bioanalytes for more than ten years because of its extraordinary sensitivity, in spite of the health risks and disposal issues posed by the use of radioisotopes.

The search for a suitable alternative to the RIA led to the development of the enzyme-linked immunosorbent assay (ELISA) in the early 1970s (Engvall and Perlmann 1971, Van Weeman and Schuurs 1971). The ELISA uses an enzymatic reaction as the basis of detection, rather than a radioactive signal. While early versions did not rival the sensitivity of the RIA, the development of highly specific monoclonal antibodies and chemiluminescence detection resulted in ELISA assays with sensitivity that exceeds that of radiolabels.

Today, key advantages of ELISA are its ease of use, flexibility, and low cost. The impact of immunoassays on life science research and clinical diagnostics has been enormous, with almost 10,000 studies published per year that include the terms “enzyme immunoassay” and “enzyme-linked immunoassay” (Lequin 2005).

Fit-for-Purpose Assays

The growth of proteomics and genomic analysis is driving the need to discover and monitor large numbers of biomarkers indicative of human disease states. The output of the Human Genome project, for example, provides the ability to simultaneously monitor the roles of multiple genes during investigations of complex biological systems.

The Bio-Plex® Suspension Array System

Suspension bead arrays provide the largest multiplexing capability for immunoassays. With xMAP bead technology, ~50 different target proteins (the theoretical limit is 100) can be simultaneously detected and quantitated in one sample. Following incubation, the sample in each well of a 96-well plate is read by the flow-based xMAP fluorescent reader in the Bio-Plex system. This platform offers not only the highest capability, but also the greatest flexibility in multiplexing according to the user’s needs. It is easy to use, inexpensive to run per analyte tested, and highly sensitive.

The Bio-Plex system is the most widely cited suspension array platform, with research applications in Alzheimer’s and Parkinson’s diseases, diabetes, obesity, cancer, asthma, cystic fibrosis, autoimmune diseases, viral infections, and vaccine development.

A wide array of assays are available, including those for the study of:

- Inflammation
- Signal transduction
- Diabetes
- Angiogenesis
- Acute phase response
- Isotyping

With the addition of the Bio-Plex Pro™ wash station, the Bio-Plex suspension array system represents an integrated solution for scientists performing high-throughput multiplex immunoassays.

Bio-Plex Assays: More Data Then — Increased Accessibility and Consistency Now

When first introduced, Bio-Plex assays accelerated research for many laboratories by generating more data points per sample in a 96-well format than other technologies could deliver. The recent introduction of the Bio-Plex Pro wash station takes this throughput to a new level of ease and accuracy by eliminating the need for additional training and producing consistent results every time an experiment is run. With this new generation of technology, if you can perform an ELISA experiment, you can run a Bio-Plex assay. Rather than perform manual wash steps after each incubation (left), the researcher simply places the 96-well plate in the wash station (right) and starts the preprogrammed wash protocol.

Prewet filter plate → **Add beads** → Wash → **Add standards, controls, and samples, 30 min** → Wash → **Add detection antibody, 30 min** → Wash → **Add streptavidin-PE, 10 min** → Wash → **Read on Bio-Plex suspension array system**

Manual Wash Method

- Place plate on vacuum manifold (any bead type), apply and set vacuum strength
- Filter
- Remove plate, blot dry base
- Add wash buffer with multichannel pipet (8 x 12 rows)
- Filter
- Remove plate, blot dry base
- Add wash buffer with multichannel pipet (8 x 12 rows)
- Filter
- Remove plate, blot dry base
- Add wash buffer with multichannel pipet (8 x 12 rows)
- Filter
- Remove plate, blot dry base
- Add next kit reagent
- Completion time varies per user

Automated Wash Method

- Place plate on Bio-Plex Pro wash station
- Select Bio-Plex Pro wash program, press start
- Add next kit reagent
- Completion time is 3 to 4 min

Recent reviews have described the power of biomarkers in the drug discovery and clinical diagnostic development processes, while also emphasizing the need to ensure that the assay is fit-for-purpose. In other words, the assay must be proven reliable for its intended use (Allinson and Brooks 2004, Lee et al. 2005, Lee et al. 2006). Thoughtful consideration must be paid to the desired goals of the experiment. When the measurement of multiple biomarkers is needed, the choice of appropriate technology typically requires striking a balance between precision, sensitivity, sample throughput, multiplexing ability, and cost. On the low-cost, low-multiplexing end of the spectrum, quantitative PCR, ELISA, and western blotting allow up to five markers to be measured simultaneously and quantitatively. On the high-cost, high-multiplexing end of the spectrum, "-omics" technologies such as microarrays, SELDI, LC/MS, and 2-D gel electrophoresis allow measurement of several hundred potential markers, but the output is essentially qualitative. The Bio-Plex platform sits in the middle of this spectrum: up to 100 markers can be measured simultaneously, while the quantitative assay performance and cost per analyte are equal to or are better than those of the low-multiplexing technologies (Figure 1).

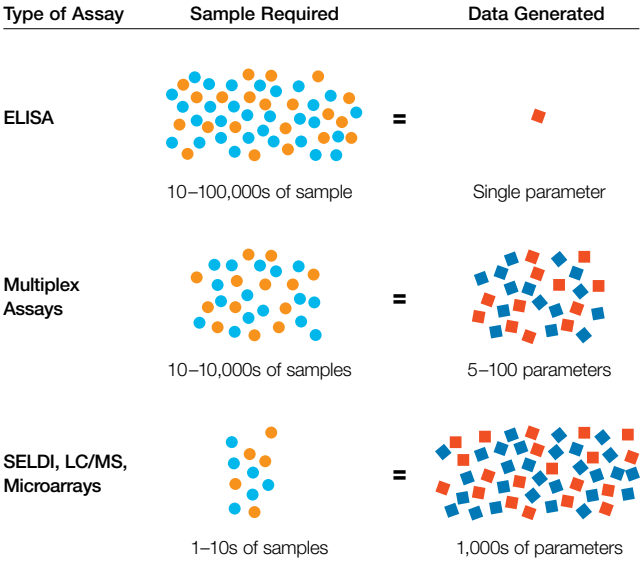


Fig. 1. Multiplex analysis and fit-for-purpose assays.

When biomarker assays are performed, it is the responsibility of the researcher to confirm that the performance of each assay is valid for its intended use in each study. Questions to assess include: Is the assay sufficiently sensitive, accurate, and precise? Does the working assay range (the lower and upper limits of quantitation) cover the desired concentration range? Are the sample dilutions required during sample preparation appropriate for the expected analyte concentrations in the samples?

Commercial developers of multiplex immunoassays therefore have two major challenges: that the assays are fit-for-purpose for the vast majority of researchers, and that the assays are fit-for-purpose in multiplex formats with different matrices (that is, with acceptably low levels of nonspecific cross-reactivity and matrix effects). Bio-Plex assays are developed using a rigorous validation process to meet these requirements.

Evolution of Multiplex Immunoassays: Automation and Magnetic Beads

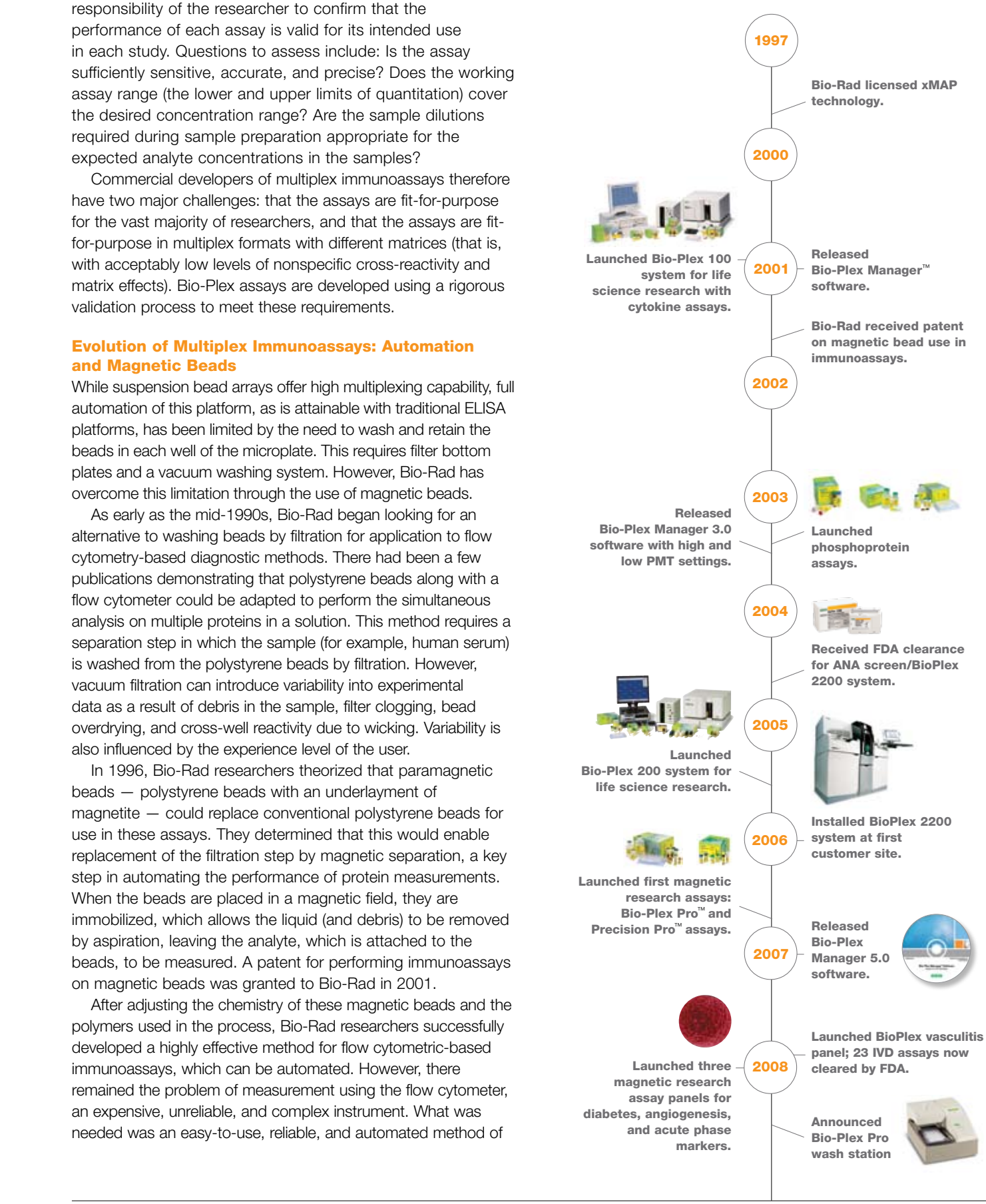
While suspension bead arrays offer high multiplexing capability, full automation of this platform, as is attainable with traditional ELISA platforms, has been limited by the need to wash and retain the beads in each well of the microplate. This requires filter bottom plates and a vacuum washing system. However, Bio-Rad has overcome this limitation through the use of magnetic beads.

As early as the mid-1990s, Bio-Rad began looking for an alternative to washing beads by filtration for application to flow cytometry-based diagnostic methods. There had been a few publications demonstrating that polystyrene beads along with a flow cytometer could be adapted to perform the simultaneous analysis on multiple proteins in a solution. This method requires a separation step in which the sample (for example, human serum) is washed from the polystyrene beads by filtration. However, vacuum filtration can introduce variability into experimental data as a result of debris in the sample, filter clogging, bead overdrying, and cross-well reactivity due to wicking. Variability is also influenced by the experience level of the user.

In 1996, Bio-Rad researchers theorized that paramagnetic beads — polystyrene beads with an underlayment of magnetite — could replace conventional polystyrene beads for use in these assays. They determined that this would enable replacement of the filtration step by magnetic separation, a key step in automating the performance of protein measurements. When the beads are placed in a magnetic field, they are immobilized, which allows the liquid (and debris) to be removed by aspiration, leaving the analyte, which is attached to the beads, to be measured. A patent for performing immunoassays on magnetic beads was granted to Bio-Rad in 2001.

After adjusting the chemistry of these magnetic beads and the polymers used in the process, Bio-Rad researchers successfully developed a highly effective method for flow cytometric-based immunoassays, which can be automated. However, there remained the problem of measurement using the flow cytometer, an expensive, unreliable, and complex instrument. What was needed was an easy-to-use, reliable, and automated method of

Evolution of the Bio-Rad Bio-Plex Magnetic xMAP Assays



testing. In 1997, Bio-Rad became aware of xMAP technology, which included a flow cytometer dedicated to the performance of multiplex bead-based immunoassays. Subsequently, Bio-Rad licensed xMAP technology for use in both life science research and clinical diagnostic applications (see the sidebar timeline and sidebar interview with Michael Barcellos). The first Bio-Plex system launched by Bio-Rad was intended for research applications, and did not incorporate magnetic beads or automation. However, many higher throughput immunoassay laboratories involved in drug development and clinical research require automation. Additionally, many automated systems utilize magnetic properties to automate sample preparation. Automating sample testing using Bio-Plex magnetic beads on a robotic sample preparation system minimizes hands-on technician time, improves precision, and streamlines workflow. Five multiplex assay panels based on magnetic beads (see timeline sidebar) are now available from Bio-Rad, and all future assays will be magnetic enabled. Bio-Plex Pro magnetic assays were developed using 25-bead map, and validated using 100-bead map xMAP technology (Figure 2), so these assays can be used by other xMAP software packages.

To facilitate automation with research assays on the Bio-Plex 200 system, Bio-Rad now offers an automated wash station (Bio-Plex Pro wash station) that can be used with both polystyrene and magnetic beads. Magnetic assays combined with the magnetic wash station help provide consistent results, regardless of user familiarity with the system and its workflow (Table 1). The addition of the magnetic wash station to the system also enhances its ease of use, making the multiplex assay workflow as simple as an ELISA. The combination of

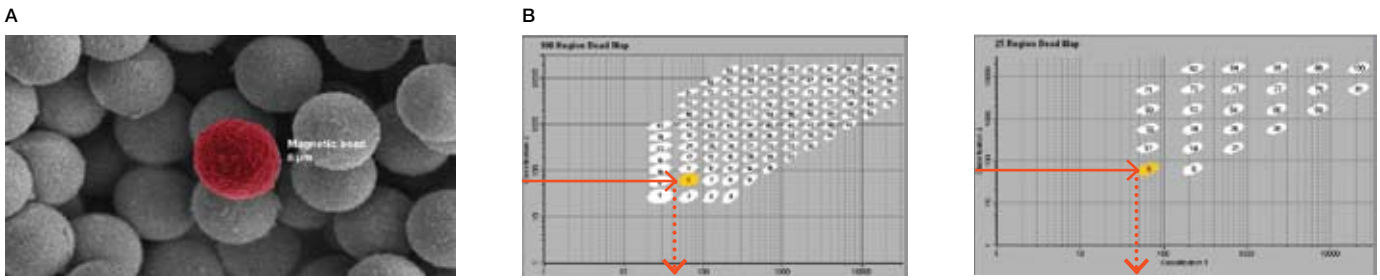


Fig. 2. Spectrally coded beads used in Bio-Plex assays. A, micrograph of 8 µm magnetic beads; each bead is labeled with a unique ratio of two fluorescent dyes. **B**, maps showing dye ratios for the xMAP 100-bead set (left) and the xMAP 25-bead set; the xMAP 25-bead region map was used to develop Bio-Rad magnetic spectrally coded beads (right). The 25-bead region map (as indicated by the arrows) is a subset of the 100-bead region map; each of the 25 regions have the identical spectral address in both maps.

Table 1. Validation of the Bio-Plex Pro wash station using the magnetic carrier and Bio-Plex Pro human cytokine assay.*

Sample	IL-1β	IL-2	IL-4	IL-5	IL-6	IL-10	IL-12 (p70)	IL-13	IFN-γ	TNF-α
%CV of median fluorescence intensity using the manual vacuum manifold										
Standard 1	1.29	0.29	1.09	1.37	0.27	1.34	0.87	1.02	1.25	2.31
Standard 2	2.2	3.37	0.87	0.53	0.1	3.52	3.87	5.03	0.71	1.95
Standard 3	1.14	7.26	3.2	4.53	2.42	1.83	3.5	4.32	4.53	1.6
Standard 4	2.65	5.01	5.54	2.61	1.47	4.12	3.22	2.38	2.09	2.44
Standard 5	1.75	7.5	7.38	5.53	1.83	0.94	0.98	1.88	2.63	4.3
Standard 6	2.91	17.33	5.53	4.13	5.48	8.87	2.2	5.38	3.76	4.82
Standard 7	1.58	13.4	5.19	1.67	2.83	2.94	3.78	8.93	2.75	12.45
Standard 8	2.65	5.38	9.29	5.03	5.5	8.28	5.68	6.95	4.78	8.57
Unknown serum sample	13.98	7.15	9.83	7.77	9.22	8.92	8.95	8.06	16.38	16.37
Observed concentration of unknown serum sample using the manual vacuum manifold (pg/ml)										
Observed concentration	6.3	Out of range	17.98	Out of range	459.06	Out of range	16.06	Out of range	30.17	13.54
Observed concentration %CV	16.04	NA	10.64	NA	10.3	NA	15.15	NA	19.25	18.35
%CV of median fluorescence intensity using the magnetic plate carrier on the Bio-Plex Pro wash station										
Standard 1	0.9	0.25	1.17	1.29	0.54	0.82	1.08	1.71	0.63	0.47
Standard 2	0.41	4.6	1.01	0.4	0.98	0.4	3.66	0.69	0.44	0.85
Standard 3	0.81	2.47	2.2	2.38	0.49	1.49	4.33	1.92	1.26	0.73
Standard 4	2.77	5	1.55	4.39	3.83	2.71	1.34	2.08	2.04	2.42
Standard 5	1.13	6.79	2.32	2.58	4.61	0.3	2.06	1.08	5.31	1.85
Standard 6	1.38	5.34	3.81	2.31	8.76	4	3.13	7.31	7.51	2.23
Standard 7	2.51	15.73	6.89	1.99	3.55	9	1.65	5.19	4.19	3.04
Standard 8	1.55	6.37	6.66	1.06	1.49	2.53	1.51	8.08	8.21	9.21
Unknown serum sample	6.59	11.48	8.25	3.73	5.64	2.33	4.79	6.19	2.77	7.47
Observed concentration of unknown serum sample using the magnetic plate carrier (pg/ml)										
Observed concentration	7.8	Out of range	14.33	Out of range	502.15	Out of range	13.45	Out of range	25.94	15.51
Observed concentration %CV	7.43	NA	9	NA	6	NA	8.92	NA	3.23	8.15

* Parallel experiments were performed by expert users to validate that the magnetic separation method achieves equal or better performance than the manual vacuum method. Results of all samples are calculated from triplicates. Standards were a serial dilution from high (1) to low (8) concentration.

Perspective on Automation of Multiplex Assays in the Clinical Diagnostic Market

Michael Barcellos, Division Marketing Manager, BioPlex 2200 Division, Clinical Diagnostics Group, Bio-Rad Laboratories, Inc.

What are the benefits of multiplexing for the diagnostic market/ customer?

Labs are looking for ways to reduce costs, minimize errors, and improve turnaround times. The ability to multiplex, that is, generate multiple reportable results from a single specimen, allows them to achieve all of these objectives. The Bio-Rad BioPlex 2200 system is the only fully automated, random access multiplex system for the in vitro diagnostics (IVD) market. With its growing menu of autoimmune and serology assays, the BioPlex 2200 system brings the benefits of multiplexing to an area of the lab that is dominated by semi-automated methods, thus bringing the additional benefit of automation, which further improves workflow.

When did the Bio-Plex 2200 system launch, and can you comment on placements?

The system was launched in late 2005, and in 2006 we had our first customer installation in North America. We now have placements in the U.S., Canada, Europe, Eastern Europe, and South Africa.

How did the Bio-Rad Clinical Diagnostics Group discover the need for magnetic beads when developing xMAP assays for IVD?

It was an issue of performance. We wanted to achieve certain levels of performance (sensitivity and specificity), and we couldn't get there with a homogeneous format. Using magnetic beads in an automated format enabled our development teams to achieve the market-leading performance we wanted in our autoimmune and serology panels.

How long from sample to result?

It is 45 minutes from first sample to first result, and every 36 seconds thereafter you get another result. The theoretical throughput with a 22-plex assay and 3 internal controls is 100 samples per hour, which equates to 2,200 tests/hr. That is how the Bio-Plex 2200 system got its name.

What assays does Bio-Rad currently offer to the IVD market?

The ANA screen (13-plex), EBV IgG (3-plex), EBV IgM (2-plex), syphilis IgG (3-plex), and the most recent release is our vasculitis kit (3-plex). All assays are FDA cleared. ToRC IgG is currently being reviewed by the FDA.

Can you mention what customers can look forward to (what's in the pipeline)?

In the area of serology, we have panels in development for syphilis IgM, ToRC IgM, MMRV-Immunity, HSV, and Lyme disease. In the area of autoimmune diseases, we are developing a gastrointestinal panel and a rheumatoid arthritis panel. We are also in development for diabetes, cardiac risk and damage, a urine toxicology test for drugs of abuse, and other longer-term projects for biomarker panels in complex diseases.

Do you see any synergies between drug discovery research applications and the clinical diagnostics labs (for example, cytokines in clinical research)?

Yes, we all share the same vision. The whole concept of developing more targeted therapies to small targeted populations and using biomarker panels to identify patients who can benefit most from drugs makes complete sense. Many pharma companies are interested in working with IVD companies to better position their drugs during the FDA submission process, and one way to do this is to bring their drugs to market with a companion diagnostic. This has yet to become a market reality, but it will happen. A number of companies have contacted us about putting their biomarker panels on the BioPlex 2200 system.

Can you share a case story where having this device has made a difference?

From a workflow standpoint, our first customer used to spend an entire day to generate the lab's daily volume of ANA results using a different (manual) multiplex product. With the BioPlex 2200 system, all the results are completed and reported out in 2 hours. This time savings underscores the real value of high-throughput and automation.

Bio-Plex Pro assays and wash station is an important step forward. These advances make the technology more accessible and reproducible — important for life science and clinical researchers under increasing pressure to produce greater quantities of reliable data.

Conclusions

Today, applications of array technologies are advancing research in genomics, proteomics, and clinical diagnostics. Suspension bead arrays provide a level of multiplex capability that cannot be matched by traditional ELISA methods. They also provide a greater degree of flexibility and higher multiplex capability than other commercially available array-based systems, at a reasonable cost per sample. The development of magnetic bead capability for the Bio-Plex system provides the benefits of multiplexing with the ease of use and sample throughput that traditional ELISA users have come to expect.

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