The A^2 MicroArray System: an open platform for multiplex immunoassay development

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Multiplexing Assays

The introduction of the microarray with its ability to globally interrogate a sample for a multitude of factors (e.g. gene expression monitoring; biomarker discovery) has quickly brought us to a new testing paradigm. The advent of "omics" driven parallel processing for quantitative biology has created new opportunities for assay development. Most attractive are turn-key platforms that offer the ability to simultaneously measure in a quantitative manner multiple analytes of interest across multiple samples, i.e. the multiplexed assay. Thus, microarrays have become very popular for the multiplex analysis of both nucleic acids and proteins. Planar slide microarrays, bead-based flow-cytometer platforms, and microplate-based array formats have been adopted for this purpose. In view of the multigenic nature of disease and the inter-relationships and complexities at the genomic, proteomic and metabolomic levels it is most certain that multiplex diagnostics and related screening technologies have a bright future. Microarray-based platforms continue to play a key role in the development of these assays.

Benefits of the Plate-based Multiplexed Assay

Multiplexed assays developed in plate-based formats (e.g. multiplexed ELISA) are able to simultaneously measure multiple analytes from a single sample in a single well. This permits the creation of disease-associated analyte panels that potentially may lead to a more accurate prognosis and/or diagnosis by the weighting of evidence derived from comparing associated biomarkers. Since all analytes can be measured using a single aliquot of the sample there is much less sample consumption. This is especially important in the case of clinical research studies when there is a limited availability of archived samples or e.g. in the quantification of excreted cytokines from cell culture media during secondary drug screening. Multiplexing, especially with device miniaturization, leads to a concomitant and substantial reduction in reagent usage. Finally, because multiplex assays are often formatted for automation, the adoption of SBS compliant array plates allows the assay to be readily available for scale up and high-throughput applications. Taking into consideration these benefits, as well as, the associated labor savings, multiplexing in this manner can lead to a significant reduction in the cost per test.

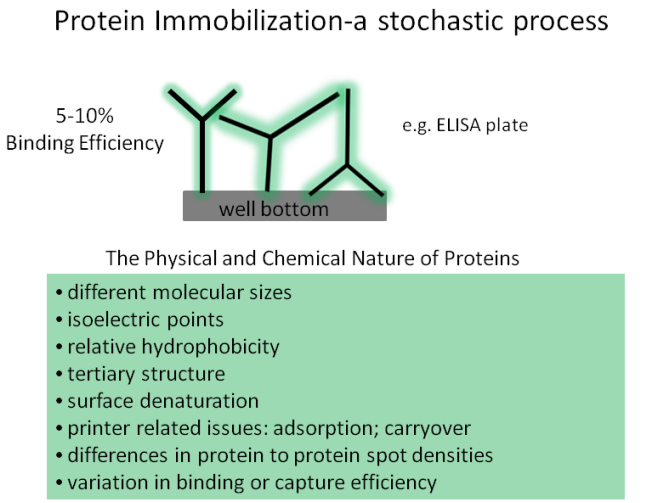
An Open Platform Approach

The microarray made its commercial debut in the mid-1990s for the purpose of scanning genomic profiles of individuals for mutation; or to monitor changes in gene expression patterns amongst control and test subjects. A decade later, the protein microarray was introduced as a tool for proteomic research. As a commercial platform, the microarray has been used largely as a *closed platform*. That is, the data or information obtained would be limited by whatever probes (the content) are affixed to the microarray by the manufacturer. So, the consumer is restricted in their experimental design to examine biological content dictated by what content menus are commercially available. An *open platform* permits the user to design microarrays with content specific for their needs. The $\mathbf{A^2}$ (A-squared) technology to be discussed here is an open platform for multiplex assay development based upon a unique microplate platform and proprietary self-

assembly chemistry that enables the user to define assays based upon their own content. Because the A^2 approach removes the need to print arrays, assay development is accelerated.

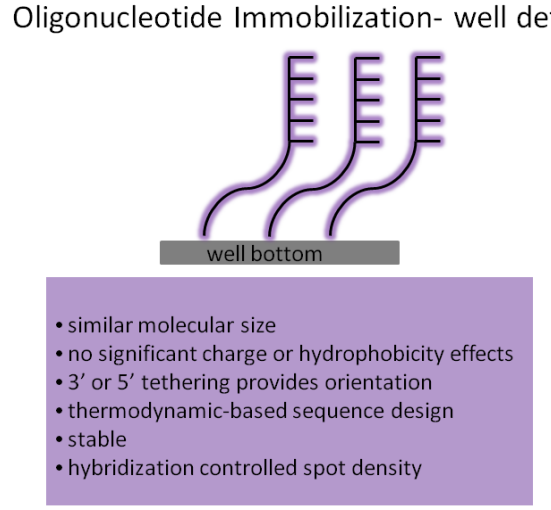
Printing Proteins Leads to Variation

The protein microarray evolved from the DNA microarray utilizing the same spotting technology to create arrays on microscope slides, the same labeling strategy and detectors. However, protein immobilization has proven to be much more problematic. Where single-stranded DNA probes (oligonucleotides) can be designed to have similar binding affinities and physical properties, proteins represent a highly diverse population of macromolecules. They vary largely in molecular size, isoelectric point, relative hydrophobicity and tertiary structure. Even within a class of proteins, such as the IgG antibodies, there can be significant differences in structure and physical-chemical stability. Proteins are often prone to surface denaturation and can differentially adsorb to various surfaces including the components of printing devices. This can lead to carryover issues during printing. Such conditions lead to differences in protein to protein spot densities. There can also be significant variation in the binding or capture efficiency of the protein probe. This is because direct protein immobilization is a stochastic process. Studies have shown that only a few percent of a protein coating participates in specific binding events. The bulk of the protein is absorbed in a random fashion and subject to surface denaturation such that binding groups are unavailable being masked or destroyed.



Using Oligonucleotides to Array Proteins

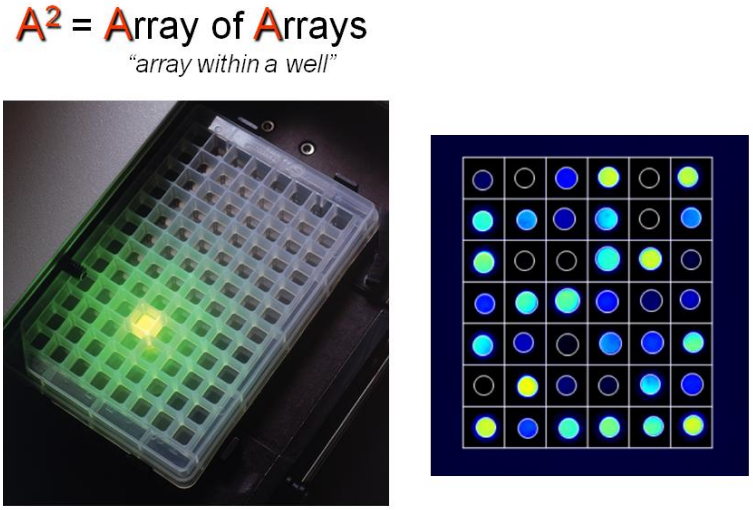
Oligonucleotides (oligos) offer some significant advantages as capture probes. They are of similar molecular size and conformation with little charge or hydrophobicity effects to contend. It is possible to tether oligos by their terminus in either a 3' or 5' orientation from the surface. More importantly, oligos can be synthesized based upon proven thermodynamic design principles to produce highly stable and well characterized reagents as probes. Printing of oligo probes is now a well developed and robust process leading to the creation of DNA microarrays of uniform spot density. Moreover, since hybridization is a thermodynamically controlled process it is possible to optimize hybrid formation of the complementary strand to the tethered oligo. Likewise, oligo-linked proteins can form hybrids in the same manner allowing for the



construction of protein arrays. Protocols for the conjugation of proteins to oligos are well known. Because the capture oligos are attached at their terminus, the complementary oligo-linked proteins are oriented away from the surface via the oligo hybrid scaffold. Binding sites on proteins are therefore less likely to be masked out by surface effects. Since hybrid density is controlled by hybridization there is less variation in protein conjugate from spot to spot. The net result is the creation of reproducible protein microarrays comprising uniform and efficient capture probes.

The A^2 MicroArray System

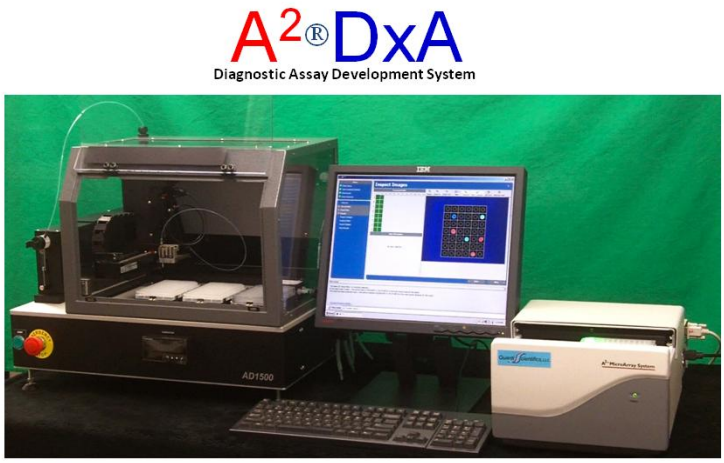
A-squared (A^2) which stands for "array of arrays" is a microplate based multiplex immunoassay platform originally developed by Beckman Coulter, Inc. QuantiScientifics now offers the *A^2 MicroArray System* which incorporates *Oligo-Link-it* technology to create protein microarrays based upon oligo-protein conjugates. A specially designed 96 square-well, polypropylene oligo capture plate, the *A^2 Plate*, is used to create 6 X 7 arrays of proteins in each well of the plate. The unique capture oligos are arranged in triplicate throughout each of the arrays. In its present format 13 different capture oligos are represented along with a reference oligo that is used to register the automated software grid tool. Thus, each A^2 plate generates 4032 data points, resulting in 1248 analyte values.



A microarray-based immunoassay for simultaneously and sensitive measurement of multiple analyte concentrations within a single sample.

The developed A^2 plate is scanned using the A^2 MicroArray System reader. The reader is comprised of a CCD camera system that incorporates up to 2 Zeiss Axio optical filter cube assemblies for dual wavelength epi-fluorescent imaging of each well bottom. An external fiber-optic tungsten halogen illuminator serves as the light source. The reader system includes an automated x,y translation stage for plate positioning, optical & positional calibration, image acquisition, data analysis and reporting. If desired, the raw image files (.tiff) can be exported and analyzed in compatible image analysis software; and data reports may be exported into Excel spreadsheets.

Recently, QuantiScientifics and BioDot, Inc. have teamed up to introduced the **A^2 DxA System** (Diagnostic Assay Development System) for automated assay development. The system includes BioDot's MDx, a customized AD1500 series non-contact dispenser which has been configured for use with the A^2 Plate providing both print function and automated assay processing. The A^2 DxA reader is configured for quality control of printed plates, as well as, imaging and assay data analysis.



BioDot MDx

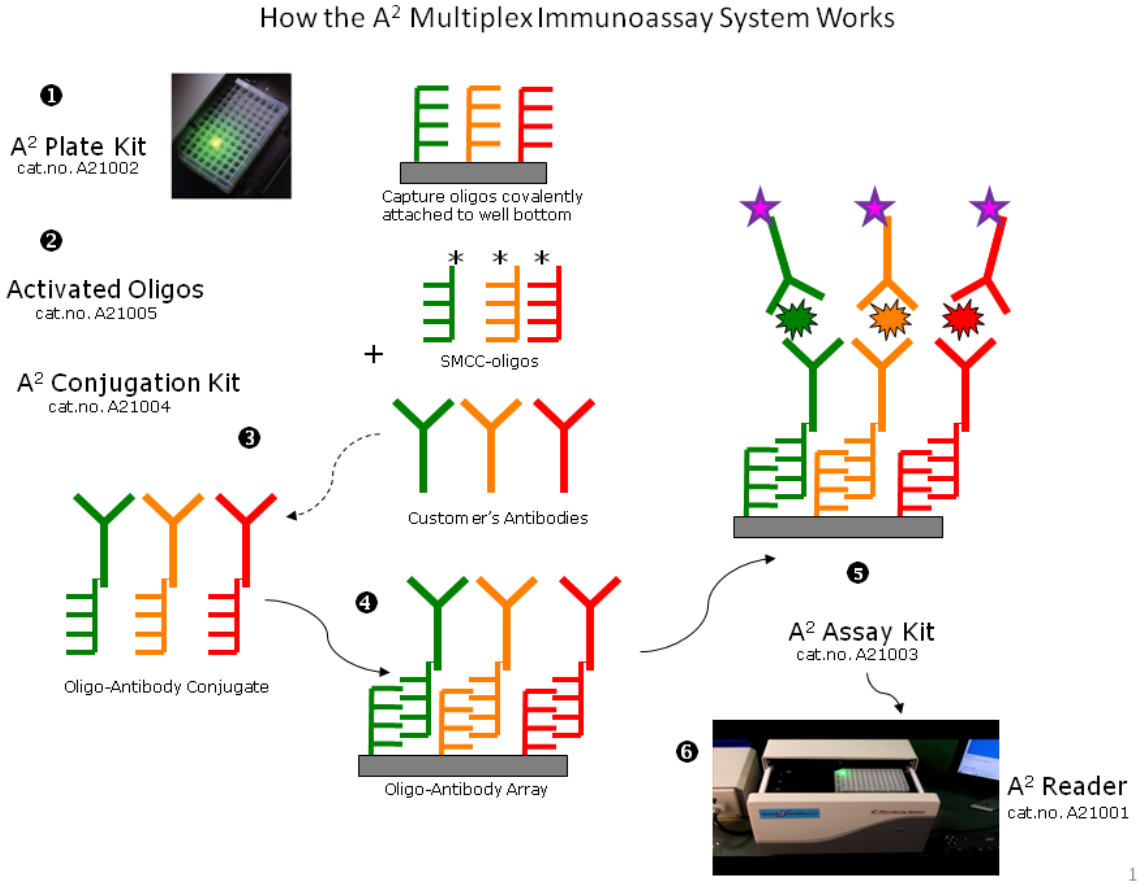
Print A^2 plate capture oligo array
Process multiplexed A^2 ELISA

A^2 MicroArray Reader

Quality Control Printing Process
Read and Analyze A^2 ELISA

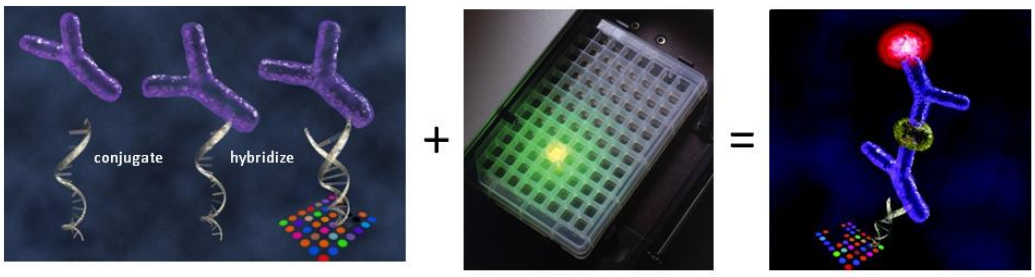
How the A^2 Multiplex Immunoassay System Works

Conjugation of Oligos and Antibodies: the user first decides how many analytes to measure in the assay. This is generally referred to as the plex-size, e.g. 5 analytes would represent a 5-plex assay panel. In that case, the user would select 5 different SMCC-activated oligos for coupling to the 5 different antibodies. Positions within the array are identified for each unique capture oligo in software (mapped) so that the user can easily define the desired assay pattern and corresponding analytes to be analyzed. The conjugation kit has been designed to conjugate 100 µg antibody using 2 OD_{260} units of SMCC-oligo. Depending upon yields and the loading concentration for the conjugate this would be enough to prepare from 5 to 15 A^2 plates. Each conjugation takes place in solid-phase using spin-columns. First, the antibody is adsorbed to the solid-phase material in the spin-column. Iminothiolane is added to generate sulfhydril groups from available lysine residues of the antibody. Finally, the SMCC-oligo is added to couple the oligo to the antibody at room temperature from 4 hours to overnight. Following coupling the residual reactants are rinsed off the column and the purified oligo-antibody conjugate is eluted ready for use.



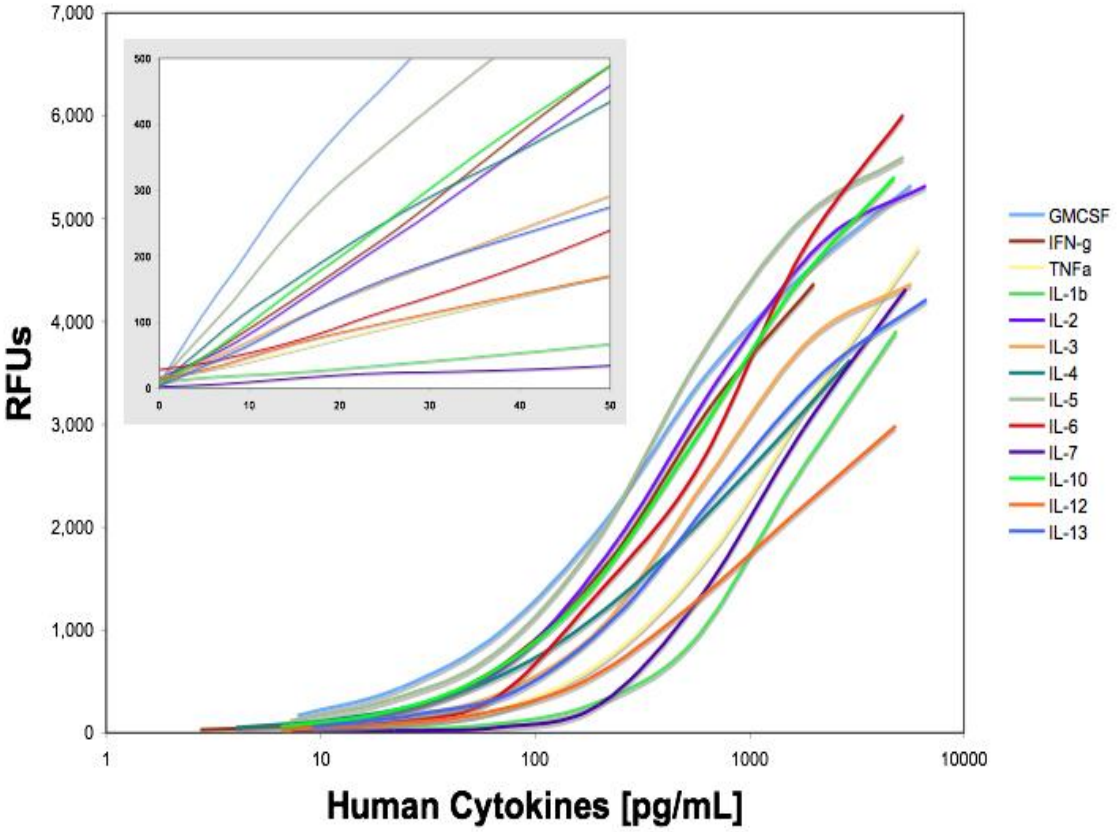
Hybridization of Oligo-Antibody: the A^2 plate is rehydrated in buffer for 5 minutes. The individual oligo-antibody conjugates are pooled in hybridization buffer and the solution applied to each well for 1 hour at room temperature. For example, oligo-antibodies are pooled at a final concentration each of 1-2 µg/mL and 55 µL (50-100 ng) applied to each well. After rinsing, the resulting antibody array plate is ready for assay. After this point, conventional ELISA or other immunoassay protocols may be used.

$A^{2®}$ Plate Technology



Creation of protein arrays by the self assembly of oligo-protein conjugates

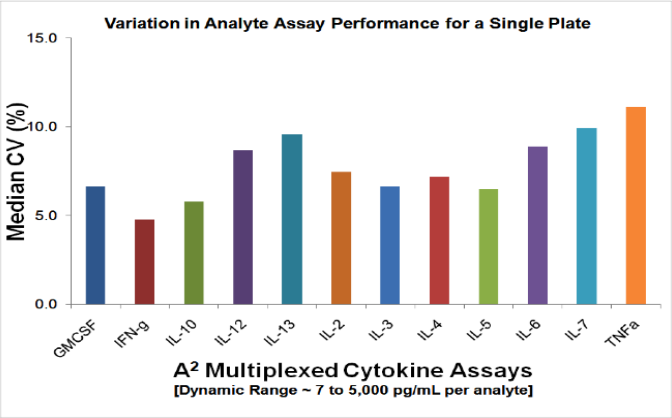
Signal Development: currently, the reader is setup for fluorescent imaging. A high sensitivity reporter based upon alkaline phosphatase signal amplification of a proprietary precipitating substrate is provided. The signal generated is read using a fluorescein filter set. Also available is a red shifted reporter, streptavidin PBXL-1 (Columbia BioSciences) permitting dual label detection. The entire plate is read within 5 minutes including data analysis and report generation. Developed plates may be re-read from 1-2 months from the initial read provided they are stored properly under refrigeration and in the dark. The user may also choose other wavelengths for analysis and purchase the corresponding Axio filter set from a third-party vendor.

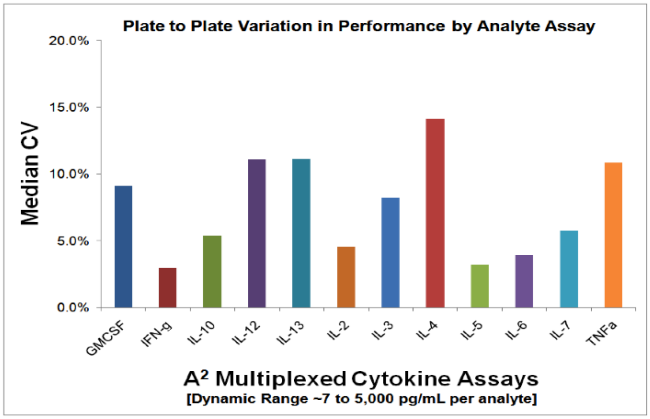


Performance

In order for an assay to be acceptable in the marketplace it first must be validated by established analytical performance criteria, especially that of sensitivity and dynamic range. For multiplexed immunoassay there is greater challenge since there is an increased potential for cross-reactivity and non-specific associations as the plex-size expands. The A^2 MicroArray System assays have undergone rigorous validation processes in order to reduce non-specific binding, cross-hybridization of oligonucleotides, and the matching of monoclonal antibody pairs to eliminate

any potential cross-reactivity. This results in the development of assays having low imprecision across a broad dynamic range. For example, the A^2 human 13-plex cytokine panel covers ~ 3 logs in dynamic range with a mean coefficient of variation (cv) across the range of between 5-10% for intra-assay for most analytes. Plate to plate (inter-assay) imprecision averages under 10% cv for this serum based assay.





IFN-g

Net Avg Int	Std Dev	%CV	Obs Conc	Exp Conc	% Recovery	Serial Dilution
4,368	208	4.8	1,992.2	2,000.0	100	Undiluted
3,194	164	5.1	671.5	666.7	101	1/3
1,674	54	3.2	219.1	222.2	99	1/9
693	30	4.4	76.5	74.1	103	1/27
225	10	4.5	23.9	24.7	97	1/81
75	4	6.0	7.8	8.2	94	1/243
31	3	9.9	2.8	2.7	101	1/729
11	7	61.4	0.2	0.0		Blank
23.6						Blank +2SD

GMCSF

Net Avg Int	Std Dev	%CV	Obs Conc	Exp Conc	% Recovery	Serial Dilution
5,327	262	4.9	6,218.3	5,680.0	109	Undiluted
4,511	313	6.9	1,720.4	1,893.3	91	1/3
3,492	185	5.3	665.6	631.1	105	1/9
2,051	149	7.3	208.1	210.4	99	1/27
999	66	6.6	68.7	70.1	98	1/81
437	34	7.9	23.5	23.4	100	1/243
169	11	6.2	7.5	7.8	97	1/729
9	6	68.6	0.3	0.0		Blank
21.6						Blank +2SD

IL-10

Net Avg Int	Std Dev	%CV	Obs Conc	Exp Conc	% Recovery	Serial Dilution
5,408	411	7.6	4,792.8	4,750.0	101	Undiluted
4,307	328	7.6	1,565.1	1,583.3	99	1/3
2,775	160	5.8	532.2	527.8	101	1/9
1,366	89	6.5	175.9	175.9	100	1/27
557	15	2.7	58.4	58.6	100	1/81
195	10	5.3	18.6	19.5	95	1/243
63	2	3.7	6.0	6.5	91	1/729
6	5	92.1	1.1	0.0		Blank
16.9						Blank +2SD

Benefits of the A^2 MicroArray Platform

Based upon a flexible, easy-to-use and standardized platform, A^2 provides more consistent results between users and laboratories. The A^2 Plate approach with self-assembly of oligo-protein conjugate reagents has proven to accelerate assay development at a reduced labor demand. The A^2 MicroArray and A^2 DxA Systems offer a "complete" solution for multiplexed immunoassays providing high precision and accuracy over a large dynamic range. The technology is scalable and automation friendly for high-throughput applications. A^2 also has broad applicability for the development of multiplexed assays for allergy testing, peptide arrays for autoimmune disease, and vaccine potency assessment, among others.