



Antigen microarrays for the serodiagnosis of infectious diseases

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Microscopic arrays (microarrays) of immobilised reagents on solid surfaces in conjunction with fluorescence detection allows high speed, quantitative data gathering. Such microarray assays have been primarily applied to the monitoring of gene expression, mutation detection and many other aspects of genome analysis. In this work, we report the use of a microarray assay for the simultaneous, quantitative determination of antibodies to *Toxoplasma gondii*, Rubella virus, Cytomegalovirus and Herpes simplex virus types 1 and 2 (ToRCH panel). Microarrays of human IgG, human IgM and viral antigens were printed on glass slides using high speed robotics. After incubation with samples and fluorescent probes the slides were scanned using confocal microscopy and quantified using the antibody dilution curves printed on the slides. Excellent agreement was obtained with enzyme immunoassay data. The test format can be incorporated in a fully automated random access immunoassay platform for routine use in clinical chemistry laboratories.

KEY WORDS: Protein microarray - Infection disease - Diagnostic method.

Immunoassays are the analytical method of choice for the detection of antigens in biological fluids either because they are the only feasible assay system or because they are the most practical and cost-effective means of obtaining accurate and precise results. Since the initial descriptions of radioimmunoassays,^{1,2} the trend has been away from liquid phase, radioactively labelled reagents towards fast, homogeneous solid phase assays.³ Other recent trends include mini-

turization of assays⁴ and the simultaneous measurement of a series or panel of antigens in a single assay.⁵

Miniturization offers the advantages of reduced reagent costs, faster kinetics and potentially improved sensitivity; simultaneous determination of antigens offers the advantage of reduced cost and faster analytical throughput. Miniturization and simultaneous determination of antigens are jointly incorporated in DNA microarray technology, a high throughput method which provides information about gene function and which has recently generated a great amount of scientific and medical interest.^{6,7} Interestingly, one of the first descriptions of such a system was for the development of antibody/antigen based tests.⁸ Automation of this approach was demonstrated by Stanford Medical School (<http://cmgm.Stanford.edu/phbrown/index.html>).

Serological tests use antibody assays for the identification of infective disease. It is well known that after an antigenic stimulus, antibodies are produced in a certain sequence. IgM antibody appears first and disappears early (typically within four weeks), whereas IgG antibody is produced later but persists longer (typically for up to twenty weeks). Thus differential detection of IgM and IgG antibodies is used to infer the time of infection and to establish the stage of the patient's infection.⁹ Primary infection

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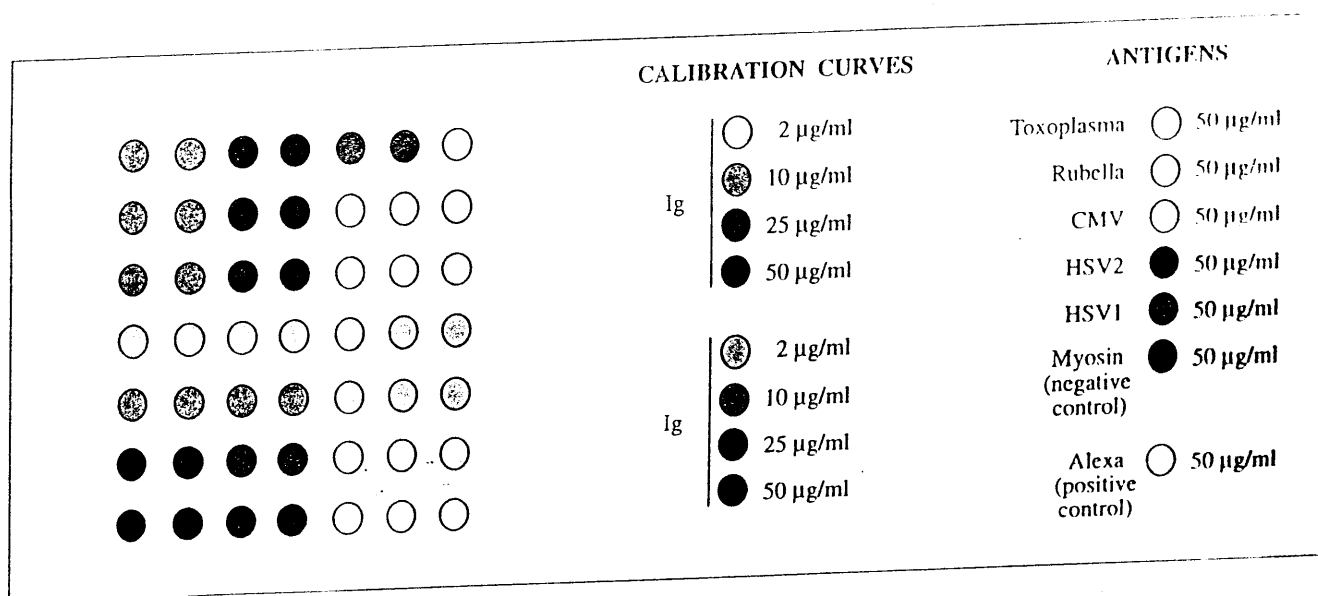


Fig. 1.—Array format.

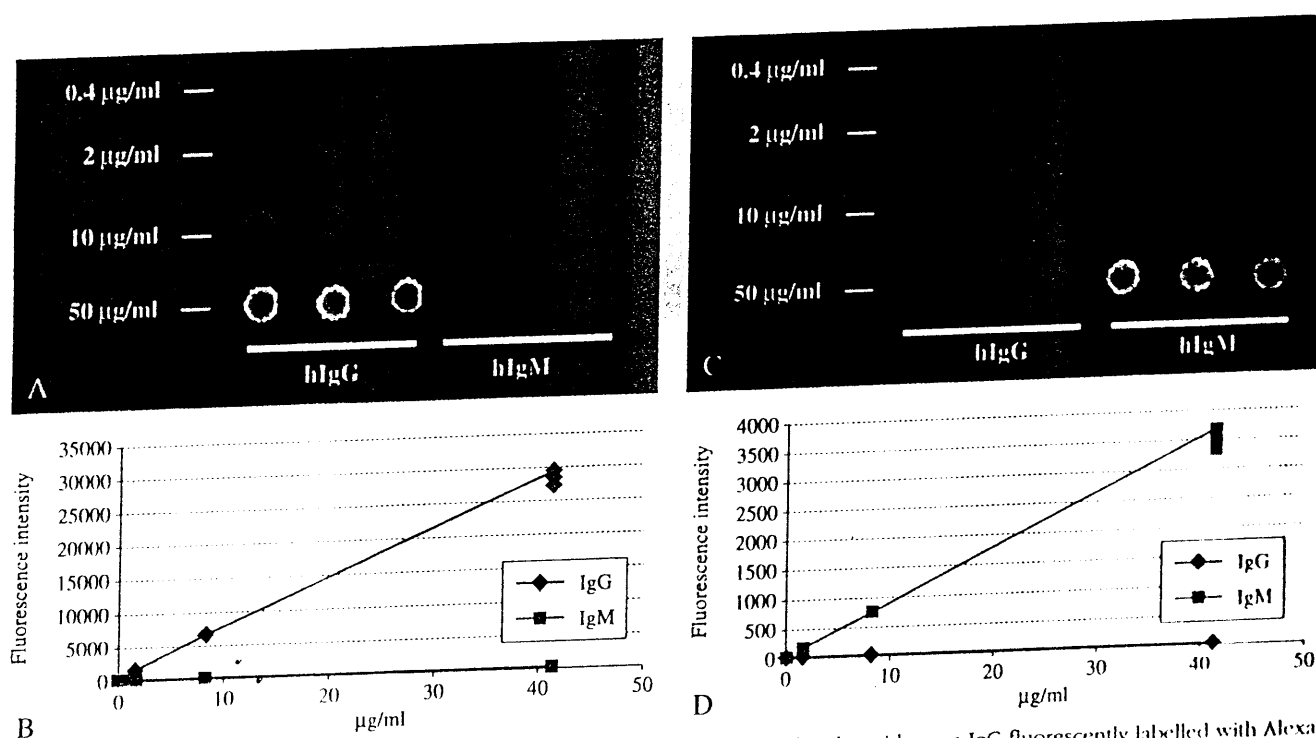


Fig. 2.—Typical microarray dose-response curves. A, B) Microarray slide developed with anti-human IgG fluorescently labelled with Alexa 546, and C, D) slide developed with anti-human IgM fluorescently labelled with Alexa 594.

with certain viruses during pregnancy can be devastating. The measurement of antibodies to *Toxoplasma gondii*, Rubella virus, Cytomegalovirus

and Herpes simplex viruses types 1 and 2 (the so-called ToRCH panel of microbes) has become routine in pregnant women. Latex agglutination and

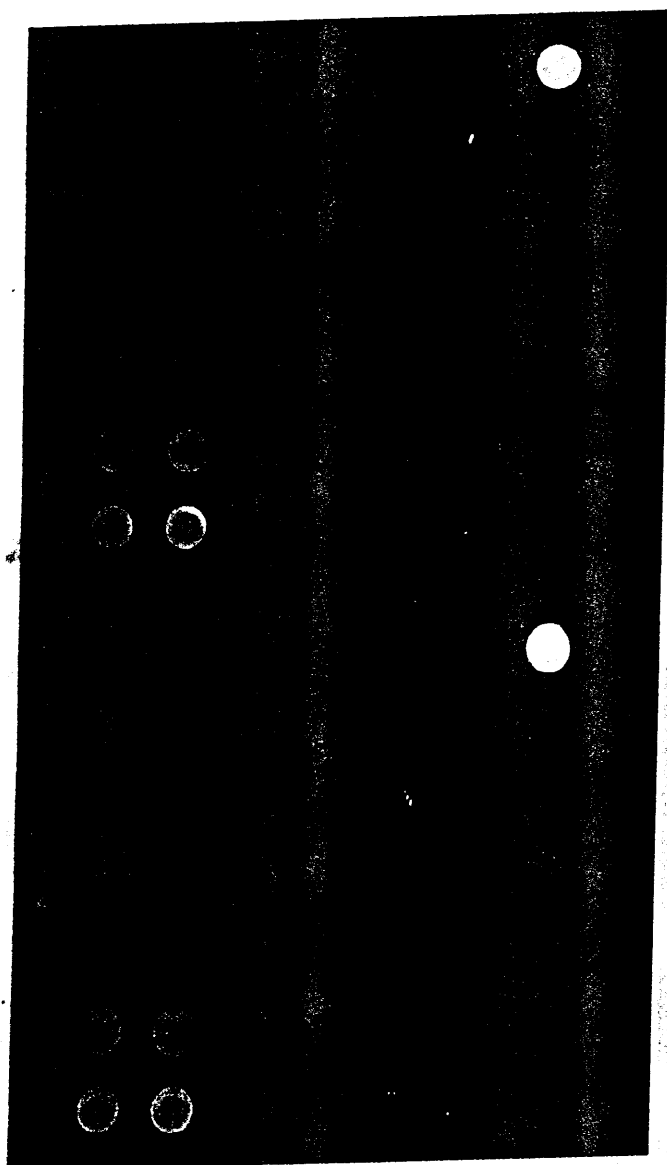


Fig. 3.—Image of microarray slide after incubated with a ToRCH negative human serum sample and developed with anti human IgG and fluorescently labelled with Alexa 546 (array printed with two pins).

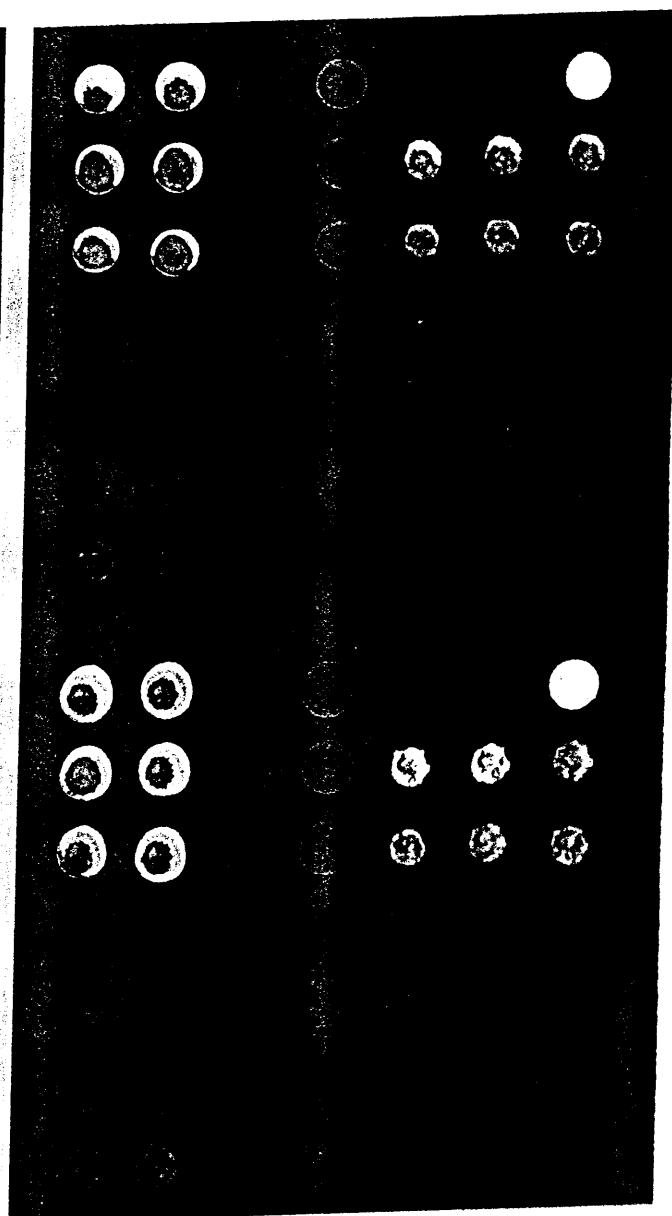


Fig. 4.—Image of microarray slide after incubated with a HSV1, HSV2, CMV, Rubella, IgG positive human serum sample and developed with anti human IgG fluorescently labelled with Alexa 546 (array printed with two pins).

enzyme immunoassay are the most widely used techniques for the measurement of these antibodies.¹⁰

The objective of this study is to use a microarray platform to develop immunoassays for the simultaneous, quantitative determination of IgG and IgM antibodies to *Toxoplasma gondii*, Rubella, Cytomegalovirus and Herpes simplex 1 and 2 (ToRCH panel).

Materials and methods

Instrumentation

A BioRobotics Total Array System (Cambridge, UK) was used for arraying. This XY axis robot can position the print-head with a high degree of precision.

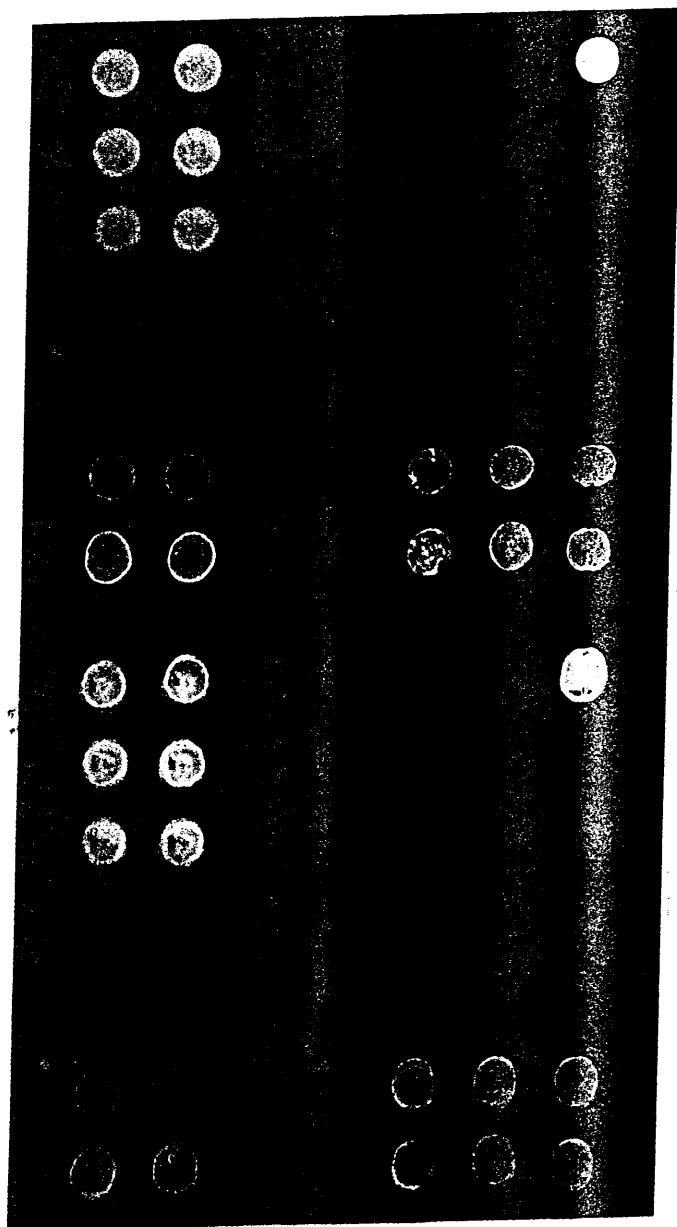


Fig. 5.—Image of microarray slide after incubated with a Toxo, Rubella, IgG positive human serum sample and developed with anti human IgG fluorescently labelled with Alexa 546 (array printed with two pins).

Key feature

The TAS combines many features (bar-code reader, temperature control, wash station, plate stacker, plate lid remover, pen design) into a compact machine (1x1x1 m). It uses 96 or 384 well plates as the sample source and microscope slides as the target. The stain-

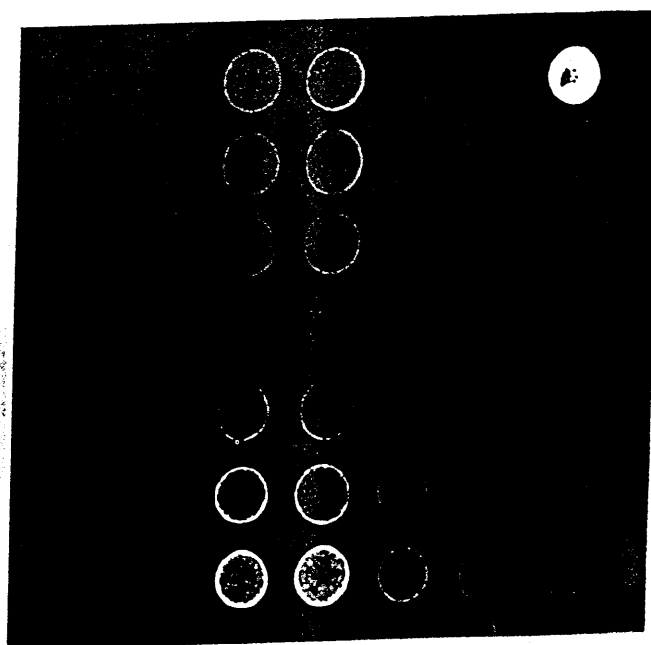


Fig. 6.—Image of microarray slide after incubated with a HSV2, Toxo, IgM positive human serum sample and developed with anti human IgM fluorescently labelled with Alexa 594 (array printed with two pin)

less steel probes or pins used to transfer sample from the source to the target are a very important factor affecting the array quality. The shape, reproducibility and durability of these pins are critical to good performance.

A General Scanning Inc. Scan Array 5000 (Watertown, Massachusetts, USA) was used for scanning the fluorescent spots.

Key feature

This instrument has four lasers for fluorophore excitation and a photomultiplier tube for light detection. It works on the principle of confocal laser scanning microscopy to provide fast, high resolution imaging of fluorescent spots.

Reagents

- Silanised microscope slides (75x25x1 mm) were purchased from CEL Associates (Houston, Texas, USA)
- 384 microtiter well plates with lids were obtained from Nunc (Roskilde, Denmark).
- Human IgG and IgM (reagent grade) were purchased from Sigma Chemical Company (St. Louis, Missouri, USA).

TABLE I.—Precision data (within-slide, six replicates per parameter) for three human serum samples (negative, equivocal, positive) analysed in a microarray immunoassay for the ToRCH antigens.

Parameter	Sample		
	Negative	Equivocal	Positive
Toxoplasma IgG:			
—signal	30.0	61.0	111.5
—SD	0.87	4.50	1.90
—CV%	2.9	7.4	1.7
Rubella IgG:			
—signal	16.7	30.0	50.7
—SD	0.60	1.69	2.93
—CV%	3.6	5.6	5.8
CMV IgG:			
—signal	8.1	27.5	73.2
—SD	0.51	0.48	6.33
—CV%	6.4	1.7	8.6
HSV1 IgG:			
—signal	6.3	77.0	124.5
—SD	0.30	3.72	5.89
—CV%	4.8	4.8	4.7
HSV2 IgG:			
—signal	4.9	14.8	49.3
—SD	0.19	0.57	1.19
—CV%	3.9	3.9	2.4

TABLE II.—Classification of human sera analysed for the serological IgG response to the ToRCH antigens by microarray and ELISA tests.

Parameter	ELISA	Microarray
	No. (%)	No. (%)
Toxoplasma IgG:		
—positive	12/56 (21.4)	11/56 (19.7)
—negative	39/56 (69.7)	41/56 (73.2)
—equivocal	5/56 (8.9)	4/56 (7.1)
Rubella IgG:		
—positive	49/56 (87.5)	32/54 (59.3)
—negative	1/56 (1.8)	19/54 (35.2)
—equivocal	6/56 (10.7)	3/54 (5.5)
CMV IgG:		
—positive	37/56 (66.1)	36/56 (64.3)
—negative	18/56 (32.1)	18/56 (32.1)
—equivocal	1/56 (1.8)	2/56 (3.6)
HSV1 IgG:		
—positive	44/56 (78.6)	43/56 (76.8)
—negative	11/56 (19.6)	11/56 (19.6)
—equivocal	1/56 (1.8)	2/56 (3.6)
HSV2 IgG:		
—positive	10/54 (18.5)	22/54 (40.7)
—negative	28/54 (51.9)	28/54 (51.9)
—equivocal	16/54 (29.6)	4/54 (7.4)

— Toxoplasma, CMV and Herpes types 1 and 2 antigens were provided by Radim S.p.A. (Rome, Italy). Rubella antigen (grade K1S) was purchased from Microbix Biosystems Inc. (Toronto, Canada). Bovine serum albumin (BSA), polyvinylpyrrolidone (PVP) and mouse serum were obtained from Sigma Chemical Company. Mouse anti-human IgG and IgM monoclonal antibodies were supplied by Radim S.p.A (Rome, Italy).

— Fluorescent Labelling of antibodies.

— Labelling of anti-human IgG was performed with Cy3 and Alexa 546 using kit reagents or protocols as supplied by Amersham Nycomed (Amersham, UK) and Molecular Probes Inc. (Oregon, USA) respectively. Anti-human IgM was labelled with the corresponding fluorophores Cy5 or Alexa 594 also supplied by Amersham Nycomed and Molecular Probes respectively.

Preparation of microarrays

Printing considerations I

Spot quality is one of the most critical factors when fabricating microarrays and can be defined by:

— volume of liquid dispensed;

— spot diameter;

— spot shape and uniformity across the entire array.

All of these parameters are influenced by:

— pin characteristics;

— composition of spotting solution;

— chemical nature of the slide surface;

— environmental conditions.

Printing considerations II

Pin characteristics:

— solid pins vs reservoir pins;

— solid pins of different materials;

— spring-loaded pins.

Custom made solid, spring-loaded pins have been used.

The sample is passively transferred to the glass slide by surface tension as the pin touches the slide surface.

Spotting affecting parameters:

— buffers;

— surfactants;

— viscosity enhancers.

Fifty mM Phosphate Buffered Saline (PBS) pH 7.2 as the base buffer usually with Tween-20 (0.01%) as the surfactant of choice. In addition the individual antigens take advantage from the addition of polymers (such as PVP or PVA at 1%) or sugars (at 5%).

Printing considerations III

Slide:

- glass *vs* membrane;
- passive adsorption *vs* covalent binding;
- chemical nature of surface.

Glass slides are preferable when used with contact printers; covalent binding gives greater stability and durability. Silanised surfaces are optimal for protein attachment.

Environmental conditions:

- temperature;
- relative humidity.

Constant monitoring of the environment during printing is recommended; we find that temperatures between 20 and 25°C and RH of 40 to 60% work well with printing of proteins.

Test protocol

Sample incubation:

- 80 µl of diluted serum sample (1 in 200) added to array;
- 15 minute incubation at RT;
- 5 ml wash buffer.

Conjugate incubation:

- 80 µl of anti human IgG-Fluorophore solution or anti human IgM-Fluorophore solution added to array;
- 1 minute incubation at RT;
- 5 ml wash buffer.

Dry slide and read in ScanArray 5000 (Figs. 1-6 and Tables I-III).

Conclusions

Good analytical and clinical performance can be obtained using microarray immunoassays.

Microarrays give significant advantages in convenience and cost when compared to traditional ELISAs.

TABLE III.—Comparison of ELISA and Microarray test protocols.

Parameter	ELISA	Microarray
Sample:		
—Dilution	1:300	1:200
—Volume (µL)	100	80
—Incubation	60	15
—Temperature (°C)	37	RT
Wash 1 (µL)	1050	5000
Conjugate:		
—Volume (µL)	100	80
—Incubation	30	1
—Temperature (°C)	37	RT
Wash 2 (µL)	1050	5000
Substrate/Stop:		
—Volume (µL)	100+100	None
—Incubation (min)	10	None
—Temperature (°C)	37	N/A
Read time (min/sample)	2	2
Signal stability	15 mins	>3 months
Total time (min):		
—1 parameter	110	20
—10 parameter	1100	20
Cost estimate (US\$):		
—1 parameter	1.5	5
—10 parameter	15	5

Microarrays lend themselves to situations where multiparametric determinations are essential such as antenatal screening, allergy testing, drug-of-abuse testing.

Microarray test format can be incorporated into a fully automated random access platform for routine use in clinical laboratories.

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