

Microarray ELISA for Autoantibody Screening in Connective Tissue Diseases

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ABSTRACT

Objective: This study was performed to demonstrate the use of an ELISA-based microarray technology which is termed as 'PictArrays', to identify autoantibody expression patterns in patients with symptoms of autoimmune connective tissue disease.

Methods: Eight commonly tested antigens were simultaneously tested on specially designed 16-well slides for their autoantibody expression patterns. The assay specificity, sensitivity and reproducibility for each of the antigens were measured. The results were analyzed by using specially developed algorithms to identify seropositive samples.

Results: The multiplex assay could identify specific antigen binding by autoimmune sera on the arrays. The PictArray sensitivity was similar to that which was obtained in established immunoassays, and the assay reproducibility was within limits

which were acceptable for diagnostic uses. The software could correctly identify the positive antigen reactivity at concentrations as low as 2 units/ml of the antibody.

Conclusion: The data demonstrated the use of a multiplex platform to simultaneously measure multiple autoimmune antibodies. PictArrays offer significant advantages over other multiplex technologies, which include (i) the use of document scanners to read the test results (ii) ease of operation which requires no specialized technical training beyond that which is required for using the conventional ELISA kits (iii) reduction in errors through software-based data analysis, and (iv) inclusion of internal controls to monitor the assay performance of each sample. These features permit the use of PictArrays in resource-constrained laboratories using existing infrastructure without significant capital expenditure.

Key Words: Microarrays, Autoantigens, Autoantibodies, Connective tissue disease, Systemic rheumatic disease, Immunoassay

INTRODUCTION

Autoimmune connective tissue diseases (ACTD) are a group of conditions which are characterized by multi-organ inflammation and autoimmunity, which affect between three to five percent of the global population [1]. These chronic diseases can be life threatening and they require immediate access to medical services during their acute phases to prevent multi-organ damage. Although the symptoms vary depending upon the disease, many diseases share the common symptoms of joint aches and pains, fatigue, muscle pain and weakness, skin rashes and the inflammation of organs [2]. Due to this, the correct diagnosis of ACTD depends not only on the clinical presentation of the patient, but on the determination of the autoantibody profile in the patients' serum.

The first and most essential step in the management of ACTD is the recognition of the disease itself by the attending physician. Since ACTD can affect any part of the body and have a myriad of clinical manifestations, an early diagnosis can result in effective treatment strategies that can arrest the autoimmune process before it can irreversibly damage the body. In the lesser economically developed regions, most physicians rely on the physical symptoms instead of using the available diagnostic tools to identify and distinguish between the various connective tissue diseases. As a result, people with ACTD often suffer for several years before being given a correct diagnosis and treatment [3].

Currently, patients who present with the symptoms of ACTD are tested for the presence of antinuclear antibodies (ANA) by doing

indirect immunofluorescent (IIF) staining of HEp-2 cells [4]. This test is inconsistent and highly dependent on subjective interpretation by the technician. The ANA positive samples are then tested for their binding to specific autoantigens, usually by ELISA. The patterns of reactivity with the individual antigens are more disease specific than ANA staining patterns, thereby providing clinically useful prognostic information [5].

As compared to the traditional ELISA tests that detect only a single analyte at a time, multiplexing provides diagnostic results for a number of different analytes on a single specimen, thus saving valuable time and money and leading to a faster diagnosis and start of treatment. The last decade has seen an increase in the commercial availability of multiplex diagnostic technologies for autoantibody screening [6-8]. However, these commercially available multiplex tests for identifying antibodies to individual autoantigens have largely been developed to meet the requirements of well-funded laboratories in highly automated environments, thus raising the cost of the testing [9]. These tests do not address the needs of a majority of the world's population in the less economically developed parts of the world, where the prevalence of autoimmune disease is similar to that in developed countries [10].

A major challenge is to develop *in vitro* diagnostic tests for use as a basis for affordable, user-friendly tests that can be deployed in the regions of the world where they are most needed. PictArrays meet the requirements for accessibility, sensitivity, specificity, reliability of results and user-friendliness which have been defined by the World

Health Organization by coining the term "ASSURED", for an ideal diagnostic test that can be used in resource-restricted settings [11].

PictArrays directly measure the antibodies which are associated with ACTD, while they also eliminate subjectivity associated with IIF techniques. Providing semi-quantitative and objective results by multiplexing changes how physicians interpret the patient's results. The need to run multiple samples from the same patient is eliminated since all the tests can be performed together for the same sample, thus providing an immediate Extractable Nuclear Antigen (ENA) profile.

The objective of this study was to use PictArrays to identify the antibody expression patterns to eight different autoimmune antigens in patients with the symptoms of ACTD. The data has been presented to show that PictArray performance was similar to that of established ELISA assays.

METHODS

Multiplex ELISA by using PictArrays

Diluted samples were added to the wells of PictArray slides [Table/Figure-1] shows the layout of the slide and the test panel), and following incubation, the wells were washed thrice with PBS which contained 0.1% Tween 20 (PBST). The sample wells were sequentially incubated with an anti-human IgG-biotin conjugate and Streptavidin-horseradish peroxidase (HRP) which was interspersed with three PBST washes. The HRP activity was measured by using 3, 3' diaminobenzidine (Thermo, USA) as the substrate and the reaction was stopped after 5 minutes by washing the wells with PBST. The dried slides were scanned on a Canoscan 5600F flatbed document scanner at 600dpi resolution. The scanned images of the coloured array spots were saved as tagged image files (*tif*) and they were analyzed by using the Pictor software.

Data Analysis

A software was developed to rapidly analyze the PictArray test results by using simple graphic user interfaces (GUI) which required minimum manual input. In brief, the protocol for the image analysis begins with the opening of the the *tif* image file and identifying the

first spot in the uppermost array of both the columns by using the cursor. The software then identifies the center of the array spots in all the wells of the slide and places a grid to measure the total intensity values of each spot. These values are corrected for background noise, based on the negative control spots within each array. The intensity values of the test spots are then corrected for non-specific signals by using the values which were obtained from well 16, which contained a sample negative control. The corrected test intensity values which were obtained within two minutes of the image capture were then used for all further analysis.

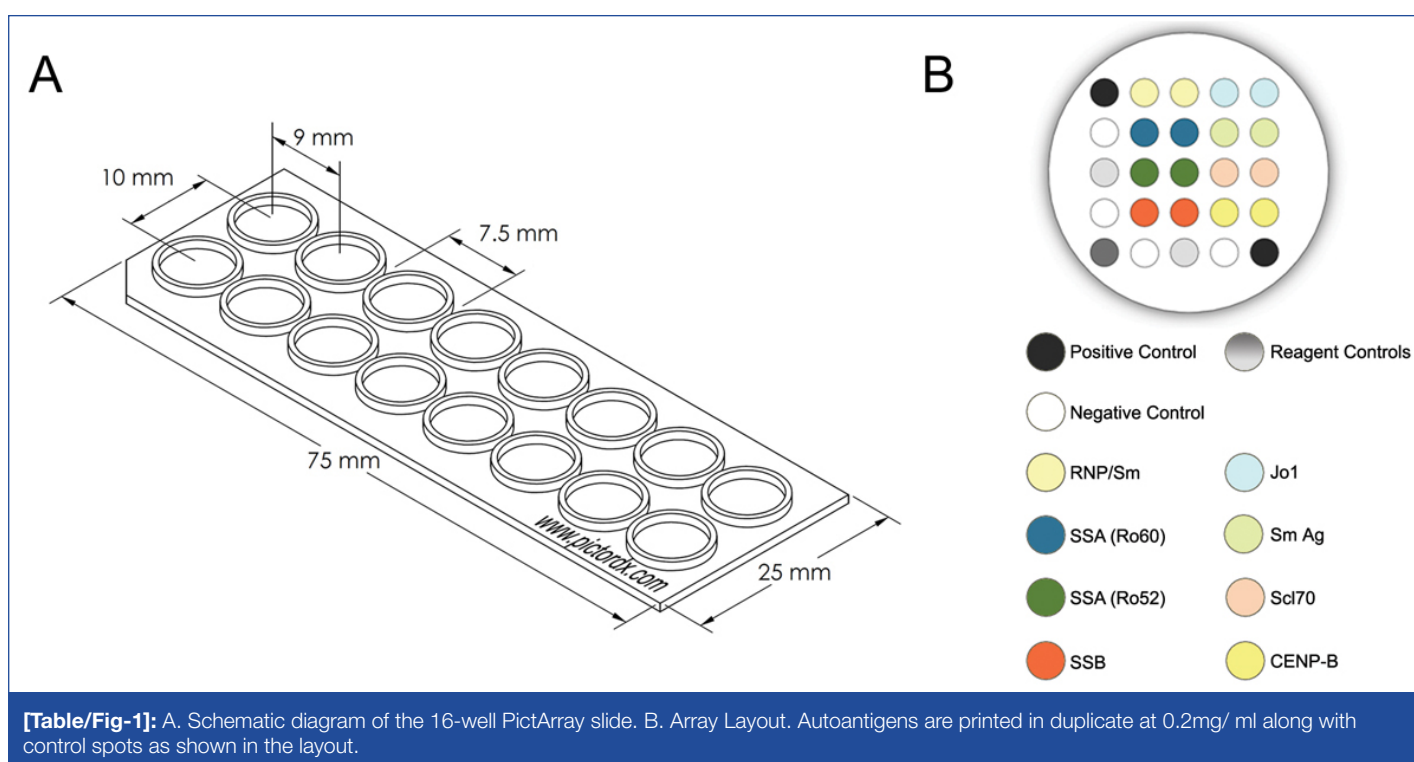
The conventional ELISA assay

Eight-well strips (Maxisorp, NUNC) were coated with 1 μ g/ml antigen in PBS and they were incubated overnight at 4°C. Step-wise incubations were performed at 37°C for 60 minutes, starting with the blocker, followed by the addition of diluted samples to the wells and washing thrice with PBST. The wells were then sequentially incubated with an anti-human IgG-biotin conjugate and Streptavidin-horseradish peroxidase, which was interspersed with three PBST washes. The horseradish peroxidase activity was measured by using 3,3',5,5'-tetramethylbenzidine (Moss, USA) as the substrate and the reaction was stopped after 5 minutes by adding 1N sulfuric acid. The colour intensity was measured by reading the absorbance at 450nm on a spectrophotometer (SpectraMax, Molecular Dynamics, USA).

RESULTS

The array layout

The panel consisted of the most commonly detected antigens, following the ANA positive testing by immunofluorescence assays [Table/Fig-2]. The 52kDa and 60kDa SSA antigens were spotted individually to improve assay sensitivity, since each antigen preparation was highly enriched for the individual component (Arotec Diagnostics, product data sheets). Each array also consisted of nine control spots which constituted the controls that monitored the performance of the sample and the reagents which were used in every step of the ELISA assay. The negative control spots are used for calculating the intra-array background.



[Table/Fig-1]: A. Schematic diagram of the 16-well PictArray slide. B. Array Layout. Autoantigens are printed in duplicate at 0.2mg/ml along with control spots as shown in the layout.

Autoantigen	Source	Likely Indication
RNP/ Sm	Calf thymus	Multiple connective tissue disease, systemic lupus erythematosus (SLE)
SSA Ro60	Calf thymus	Systemic lupus erythematosus (SLE), Sjögren's Syndrome
SSA Ro52	Recombinant	Systemic lupus erythematosus (SLE), Sjögren's Syndrome
SSB	Calf thymus	Systemic lupus erythematosus (SLE), Sjögren's Syndrome
Jo1	Calf thymus	Myositis
Sm	Calf thymus	Systemic lupus erythematosus (SLE)
Scl70	Calf thymus	Diffuse scleroderma
CENP-B	Recombinant	Scleroderma (limited form), CREST syndrome

[Table/Fig-2]:

Source of autoantigens used in the array and connective tissue disease implicated by presence of specific autoantibody.

Assay specificity and sensitivity

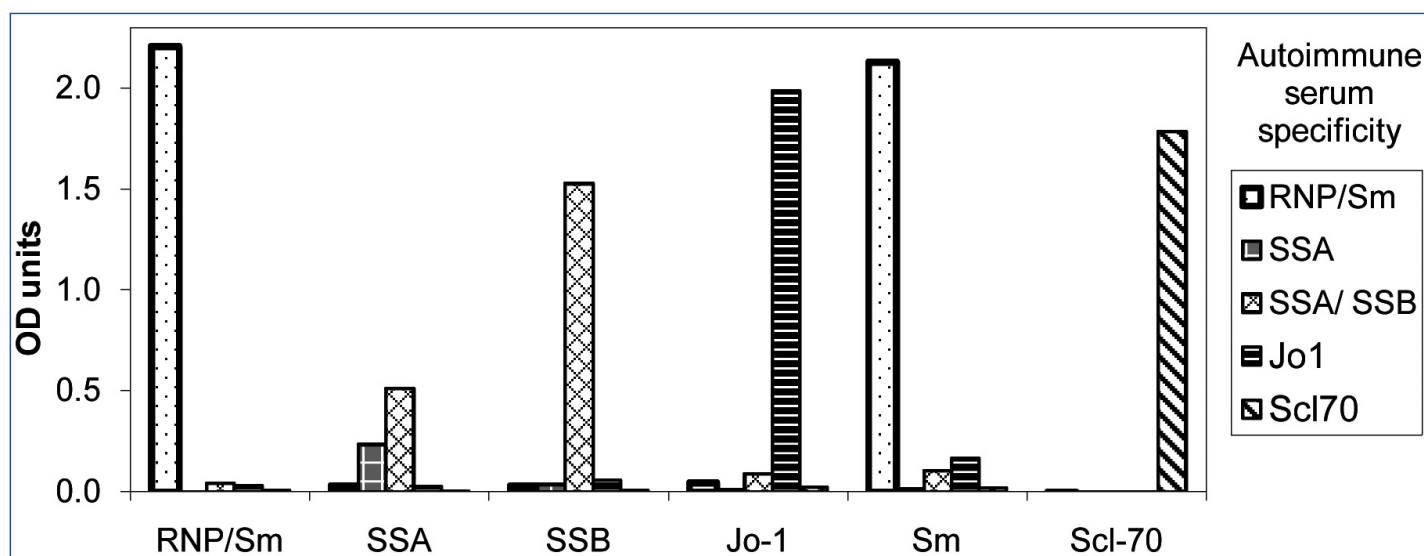
Serum samples which were positive for specific auto-antigens were the kind gift of Dr. Neil Cook (Arotec Diagnostics Limited, New Zealand). These samples were retested by using commercially available ELISA kits (Diagnostic Automation, CA) to confirm the autoantibody specificity [Table/Figure-3]. Since the kit did not contain wells which were coated with the CENP-B antigen, the CENP-B positive sample was not independently tested. No detectable cross-reactivity was obtained for antibody binding to any of the printed antigens [Table/Figure-4].

The sensitivity of the autoantibody detection to the PictArrays was determined by measuring antigen binding at a range of serum dilutions which started from an initial 100-fold dilution. Detectable antibodies to specific antigens were seen for all the samples which were tested (data not shown). However, in order to compare the array results with those from conventional ELISA, the sera had to be diluted 2-fold from an initial 1000-fold dilution. The results showed an array sensitivity comparable to that which was obtained by the conventional ELISA [Table/Figure-5].

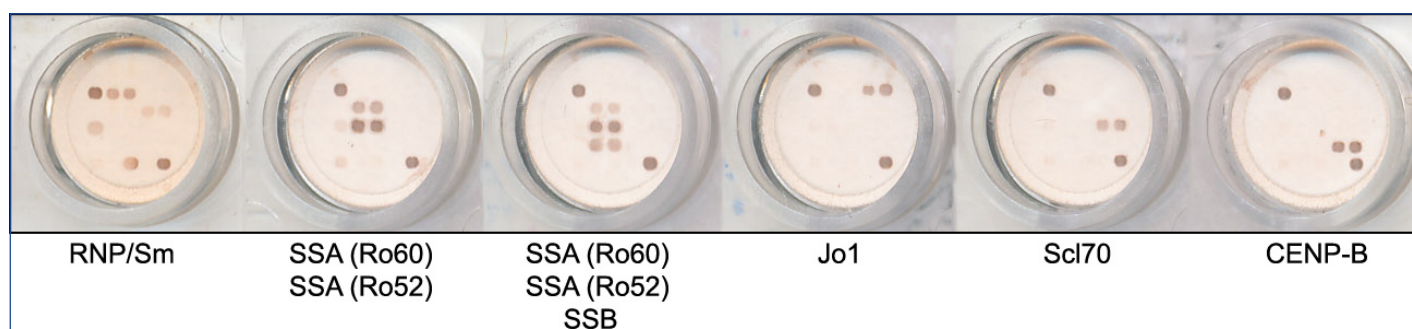
In an effort to measure the amount of antibody which was bound to the arrayed antigens a standard curve was generated by titrating known concentrations of human IgG on the arrayed anti-human IgG spot in four wells of the slide. The results showed that the antigen-specific antibody at levels of 5ng per ml of serum could be detected on the autoantibody arrays (data not shown).

Software-based Identification of Sera Containing Autoantibodies

One hundred and twenty five serum samples from healthy subjects (Sera Labs, UK) were tested on arrays to establish the threshold levels of serum antibodies to autoantigens in the general population. The average signal intensity values which were obtained from the healthy cohort for each antigen was added to 1.6 times the standard deviation to calculate the threshold. The samples were reported as negative if the signal intensity fell below the threshold value. Samples with intensity values between the threshold and two times the threshold were reported as



[Table/Fig-3]: Confirmation of auto-antigen specificity. Antigen-binding specificity of autoimmune sera on a commercial ELISA kit was tested following the manufacturer's instructions (Diagnostic Automation, USA). Each of six autoimmune sera specific for the antigen shown on the x-axis was tested in an 8-well strip. Six wells were coated with individual antigens, while one well each served as a positive and negative control.



[Table/Fig-4]: PictArray specificity. The array image shows binding of antibodies to specific autoantigens in 400-fold diluted samples (refer to the array layout in Figure 1B). Autoantigen specificity of sera is specified below image of each well.

ambiguous (+/-), while those which were above two times the threshold were positive for the presence of an autoantibody to the specific antigen. By using this algorithm, a positive reactivity could be detected at less than 2 units/ml when standards with known autoantibody units were tested on the arrays [Table/Figure-6].

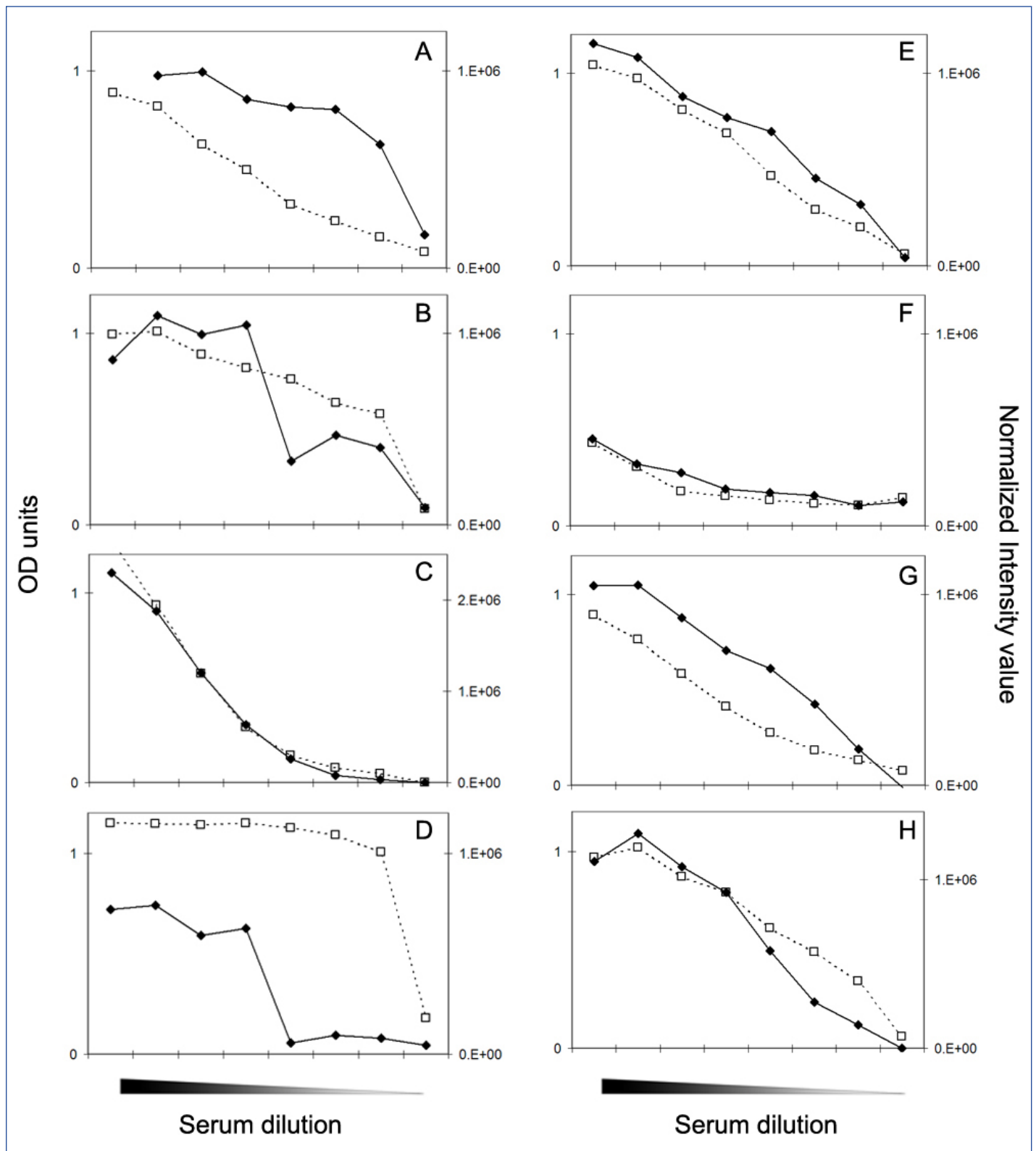
Assay Reproducibility

Fifteen replicates of each autoantigen-specific positive sample were tested to determine the inter-array coefficient of the variation.

The results showed less than 20% variability in the normalized signal intensity [Table/Figure-7] at a 400-fold serum dilution. This level of variability did not affect reporting of test results as being either positive or negative for antibody binding to the arrayed antigen.

DISCUSSION

Autoantibodies are central to the diagnosis and assessment of autoimmune connective tissue diseases. Several reports have



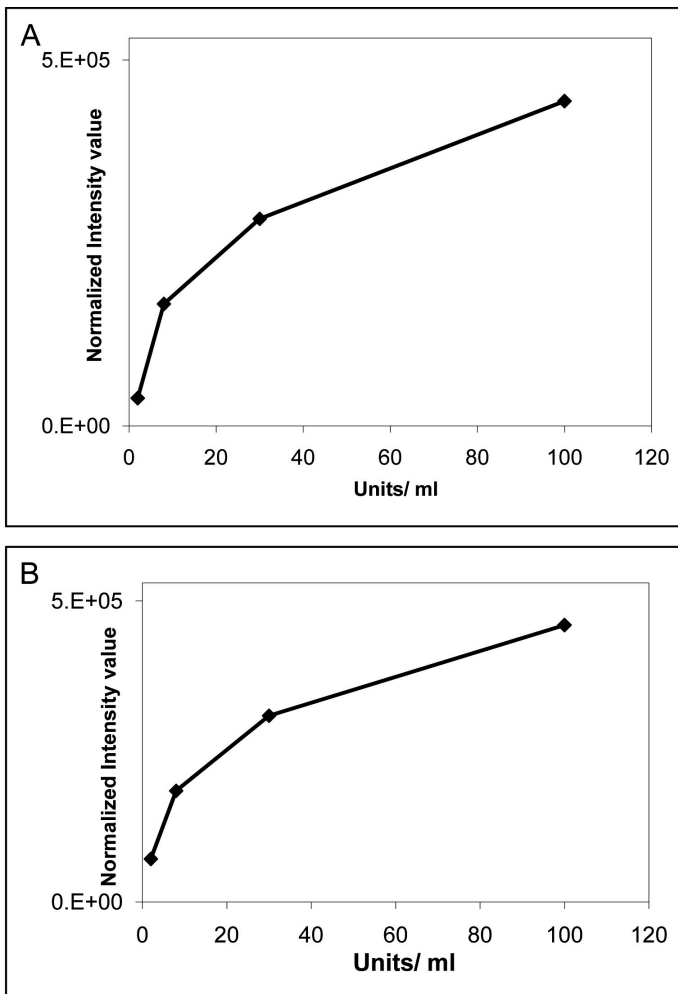
[Table/Fig-5]: Assay sensitivity. Comparison of results obtained from testing serum samples at a range of dilutions on arrays (♦) and conventional ELISA (◻). A two-fold serial dilution of serum samples were tested from an initial thousand-fold dilution on arrays as well as 8-well strips (Maxisorp, NUNC) coated with individual antigens as described in the Methods section. A. RNP/ Sm; B. SSA (Ro60); C. SSA (Ro52); D. SSB; E. Jo1; F. Sm Antigen; G. Scl70; H. CENP-B.

shown complex autoantibody patterns in patients with connective tissue diseases and they have suggested the need to screen several tens of autoantigens to determine specific antibody patterns [12]. However, interpretation of complex data on the reactivity to tens

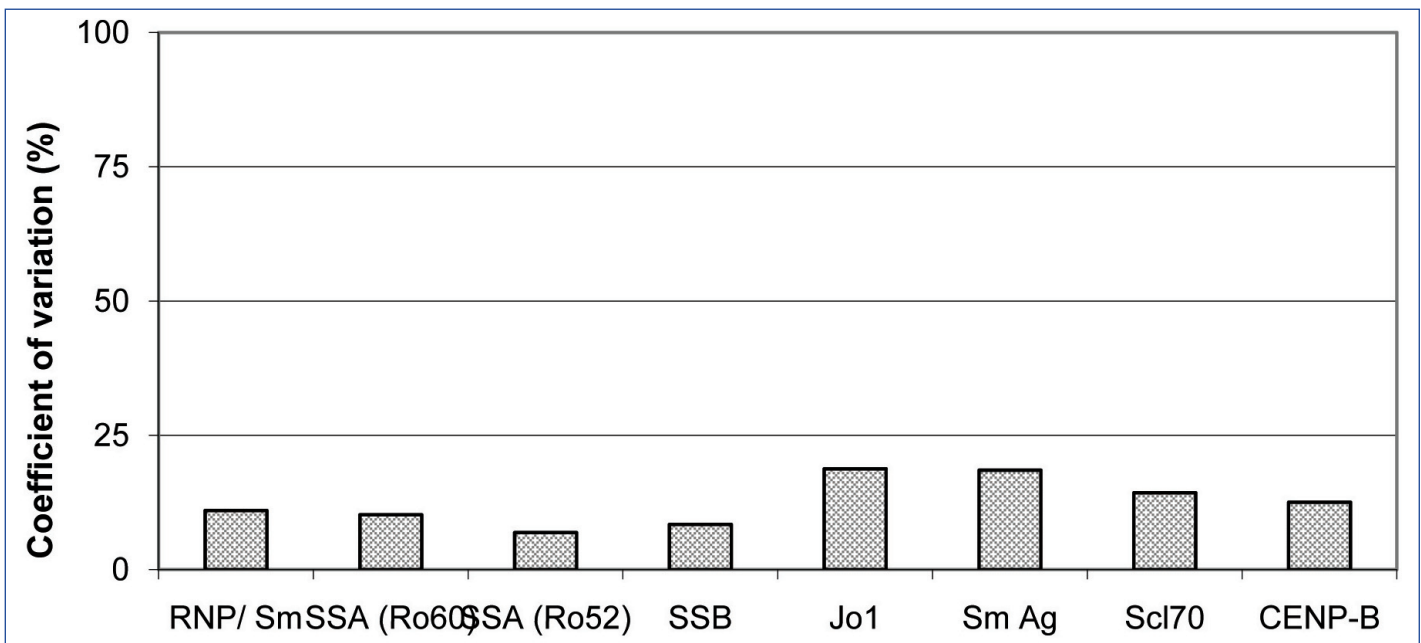
of antigens by rheumatologists and its practical importance in making treatment decisions remains to be demonstrated. The commonly followed algorithm for the diagnosis of autoimmune connective tissue diseases is based on the scores which are derived from the physical symptoms and ANA testing by IIF, which is considered to be the gold standard for ANA screening [13]. A positive ANA test is followed by testing for specific autoantigen binding [4]. The disadvantages of IIF include a high demand on laboratory personnel time, difficulty in standardization of the assay method due to variations in substrate and sample processing, and most importantly, the subjective interpretation of the results by pathologist [14].

The ENA screen as an ELISA test was introduced into clinical practice to permit an initial evaluation of the presence of antibodies to any extractable nuclear antigen before performing individual tests [15]. The inability of IIF in detecting rare antibodies against centrioles and other cellular targets was compensated by the higher sensitivity for the detection of antibodies to some antigens, which could have been missed by IIF [16-18]. Maguire et al [19] reported a study in which patients who tested positive for ANA by IIF and negative by ELISA were indistinguishable in symptoms from patients who tested negative by both assays after a one-year follow-up. Their study suggested that ELISA would reduce the number of patients who were referred to a specialist and were subjected to needless follow-up. It has also been suggested that when there is a high clinical suspicion of connective tissue disease, a focused testing for specific autoantibodies should be performed, irrespective of the ANA result [17].

As an alternate approach, multiplex assays overcome many of the shortcomings of IIF while enhancing the value of traditional ELISA tests [20]. They can be used to rapidly screen and concurrently characterize a wide range of autoantibodies. Additionally, the analysis of the test data by computer-generated algorithms reduces subjectivity in interpretation of the results. The ability to include internal controls in every sample in order to monitor the test performance reduces the chances of human and mechanical errors as compared to a conventional ELISA assay which is done by using one well-one test microtiter plates. Several studies



[Table/Fig-6]: Clinical sensitivity. The minimum detectable level of antibody binding to autoantigens was determined by testing control standards at four different concentrations. The relationship between the standard concentrations given as Units/ ml and the normalized intensity values is shown for two of the antigens. A. RNP/ Sm; B. SSA (Ro60).



[Table/Fig-7]: Inter-assay reproducibility. Samples positive for each arrayed autoantigen were tested in fifteen replicate wells at a 400-fold dilution to measure the coefficient of variation.

which were performed over the last several years have shown a correlation between the results of the multiplex and ELISA [21]. Autoantigen arrays have been suggested for use in following autoantibody profiles over time as the markers of disease remission and relapse.

Most commercially available multiplex systems have significant setup and operational costs, which restrict their use to large, highly resourced and automated laboratories in regions with high labour costs. A report which compared the performance of various commercially available ELISA assays for autoantibody screening indicated that no single vendor provided tests that are vastly superior to the other, so that the ultimate selection of the assay system may be determined by factors such as differences in cost, customer service and turn-around time [22].

We have designed 16-well disposable polycarbonate slides which can be reproducibly manufactured in large quantities, which require picogram amounts of antigens to be immobilized in the array spots. These slides permit the use of standard laboratory instruments for the sample processing and are also conducive to rapid scale-up by using commercially available ELISA analyzers. Pads of high-protein binding membranes which are laminated with a double-sided adhesive are inserted into the wells of these slides. Arrays of the autoantigens are spotted onto these pads, following a standardized layout in which a 5x5 array with control spots in the first column and in the last row of each array are included to monitor every step of the ELISA; controls confirm the integrity of the serum sample as well as the performance of the detection antibodies and the enzyme substrate. Duplicate spots of the eight tests complete the array. This layout can be maintained for all the ELISA-based tests with minor variations, to account for the types of tests which are included in the panel. The use of a precipitable peroxidase substrate that results in the deposition of the coloured product on the array spot enables the use of a flatbed scanner for reading the test results. This reduces the setup costs significantly, allowing access to a multiplex technology in those regions where its need is highest. The development of proprietary software to convert the image file into a clinical result within two minutes of test completion enables technicians in a clinical diagnostic laboratory to rapidly obtain test results with minimum data handling. Moreover, the test results can be obtained as a text file, which can be easily integrated into the existing laboratory databases for storage and reporting, in formats which are developed by the individual laboratories.

We suggest that the PictArray ENA panel is a viable replacement for the individual ELISA assays. PictArrays can satisfy the need for both an initial screen, as well as for the subsequent determination of clinically significant autoantibodies. We have demonstrated that the performance of PictArrays was similar to that of established ELISA assays for each of the eight antigens which were tested. The array results demonstrated excellent analytical specificity and sensitivity, while software-based algorithms for the identification of samples which contained specific autoantibodies provided rapid test results.

In conclusion, we present data for an affordable multiplex platform in which multiple biomarkers can be measured simultaneously. This technology can be easily integrated into diagnostic laboratories with a moderate level of resources and infrastructure. Further developments are underway to enable the use of this technology at the point-of-care. PictArrays offer significant advantages over other multiplex technologies, which include, (i) low setup and operational

costs due to lack of the requirement for sophisticated instruments, (ii) easy operation which requires no specialized technical training since the assay is based on ELISA, (iii) reduction in errors due to a software based data analysis and the inclusion of internal controls to monitor the individual assay performance.

Autoimmune diseases are on the rise due to an aging population and coupled with the need for an extensive infectious disease testing, multiplex technology is a timely and much needed addition to the clinical diagnostic laboratory. There is a real need to include many different types of tests on one platform (such as infectious diseases and autoimmune diseases) to save space, time and money, and to limit medical waste. Performing ELISA tests in a multiplex panel as has been reported in this paper by using PictArrays, combines an up and coming testing format with a tried and true testing method. As the PictArray test menu increases, the potential cost and the throughput benefits which are realized by the laboratory will increase exponentially.

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DISCLOSURE STATEMENT:

The Authors are Employees of Pictor Limited.

Date Of Submission: **Oct 09, 2011**

Date Of Peer Review: **Jan 05, 2012**

Date Of Acceptance: **Jan 12, 2012**

Date Of Publishing: **Apr 15, 2012**