

Development of a Low-Cost, Colorimetric Protein Array System to Measure Human Autoimmune Disease Markers

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A sensitive low-cost method for producing and visualizing protein microarrays was developed. This method utilizes a protein-specific microarray substrate that immobilizes proteins, therefore allowing for the detection of protein-protein, protein-antibody, and protein-drug interactions. This protein array substrate, termed ζ -grip, has been optimized to work with high sensitivity protein detection chemistries and with a nonfluorescent, low-cost, image-based detection system. Using this system, we validate the application of ζ -grip in the analysis of autoimmune disease markers by screening patients diagnosed with autoimmune disorders, illustrating the feasibility, sensitivity, and repeatability of the above-mentioned substrate and system for this purpose.

INTRODUCTION

The recent completion of the human genome sequence has caused a surge of interest in “solving” the human proteome (i.e., finding methods to study and monitor the activities of native proteins). Past and current methods entail procedures such as enzyme-linked immunosorbent assay (ELISA) testing, Western blot analysis, radioimmunoassays, 2-dimensional gel electrophoresis, protein-protein interactions by two-hybrid systems, co-immunoprecipitation, and protein affinity chromatography (1–8). Unfortunately, high amounts (4 to 5 orders of magnitude greater than the ζ -grip method) of costly antigens and patient samples are required in ELISA testing, Western blot analysis, and radioimmunoassay (2,9,10). Some methods have been described as tedious, not high-throughput, low sensitivity, and having limited control over experimental conditions (11–13). Protein array-based techniques are, therefore, likely to replace these methods by easing the problems of previous and current methods, and will allow more advanced medical diagnostics. A number of research groups are already working on protein array chip technology. To date, there are techniques involving protein scaffolds and aptamers to capture molecules; fluorescence resonance energy transfer, surface plasmon resonance, and potential difference measurements to detect proteins; and polyacrylamide gel, layered membranes, nitrocellulose, hydrogel, and silanated glass with epoxy, aldehyde, and amine or lysine coats to act as the array substrate surface (13–19). There are still a number of obstacles to overcome for protein microarrays to find widespread use as a diagnostic and research platform. For example, recently developed procedures are not able to detect a number of autoantigens when epitopes are lost upon denaturation, electrostatic repulsion, or steric interference (20). There are also issues related to substrates. Silanated glass slides were originally developed for DNA microarrays

and do not give consistent results for protein immunochemistry. In addition, some slide coatings were prone to shrinking and diffusion yielding undesirable effects.

The focus of this paper is on a method that uses the “ ζ -grip,” a new protein microarray substrate that allows protein immobilization for protein-interaction analyses, together with a low cost scanner, which finds an obvious application in the detection of autoimmune protein biomarkers. Typically, autoimmune diseases are difficult to diagnose, especially in the early stage of disease, since many symptoms are nonspecific, and current laboratory tests are inadequate to confirm a diagnosis. One method for detecting protein markers of autoimmune diseases is through ELISA testing—a rapid test where an antibody or antigen is linked to an enzyme, as a means of detecting a match between the antibody and antigen (1). By designing a protein microarray substrate based on the concept of ELISA testing, such as the ζ -grip, many disease markers can be measured at the same time. This is particularly important for autoimmune diseases such as systemic lupus erythematosus (SLE) and Sjögren syndrome (SS). The disease markers investigated in the present study are briefly described below.

DISEASE MARKERS

Sjögren's Syndrome Type A (SSA) and Sjögren's Syndrome Type B (SSB) Antigens

Autoantibodies to Ro/SSA and La/SSB cellular antigens are commonly found in the sera of patients with several autoimmune diseases, including SS, SLE, neonatal lupus erythematosus (NLE), subcutaneous lupus erythematosus, and rheumatoid arthritis (RA) (21). The Ro/SSA antigen is comprised of an acidic 60-kDa protein that may be associated with RNA, ranging in size from 80 to 112 bases, and the presence of autoantibodies against it may be used as a

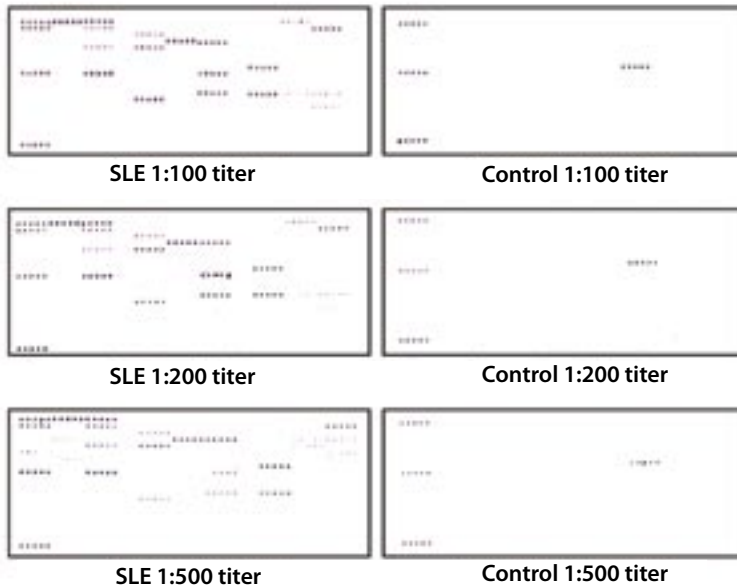


Figure 1. SLE multianalyte microarray panel results. Results of six arrays using a pool of five SLE patients' serum, or a pool of five corresponding age- and sex-matched control patients' serum, for a range of titers. Positive responses can be traced back to the original antigen in the 384-well dish, identified in Table 1. Positive control (Control +ve) for calibration is located at A1, A9, A20, and G9, and is used as a guide when the developing process is complete. All other antigens were diluted as pure, 10/30, 5/35, and 1/39 (μL sera/ μL PBS).

diagnostic tool (22). Increased levels of anti-Ro/SSA have been detected in as high as 96% of patients with SS, 49% of patients with SLE (23), and 83% of mothers of infants with isolated complete Lupus.

Mitochondrial Antigen

Primary biliary cirrhosis (PBC) is an autoimmune disease, characterized by inflammation of the septal and interlobular bile ducts. Anti-mitochondrial antibodies (AMA) are very closely linked to PBC and can be detected in more than 95% of these patients (24). The molecular cloning and expression of antigens of the ketoacid dehydrogenase complex has contributed to the diagnosis of AMA-negative PBC and is an important marker in overlap syndromes of PBC and other autoimmune liver diseases (25). AMA reacts with members of the 2-oxoacid dehydrogenase complex (2-OADC), predominantly binding to conformational epitopes of the inner lipoyl domains of the highly conserved E2 subunit. Over 95% of PBC patients have antibodies reactive with the E2 subunit of pyruvate dehydrogenase complex (PDC-E2), which is considered to be the major autoantigen (26). In fact, AMA has been found to be detectable in peripheral blood many years before the onset of clinical, biochemical, or histological features of the disease (27).

Sa, IgG, IgM, and Histidyl tRNA Synthetase Antigens

Anti-Sa antibodies have been described to be highly specific markers for rheumatoid arthritis (RA). At least 2 different subsets of autoantibodies are present in anti-Sa sera, one directed against a 68-kDa Sa protein and another to the typical 50/46 bands of the Sa immune system (28).

In 1985, a group of investigators found a strong association between anti-neutrophil cytoplasmic antibodies and active Wegener's granulomatosis (29). Immunoglobulin G (IgG) autoantibodies against extranuclear components of polymorphonuclear granulocytes were detected in 25 of 27 serum samples from patients with active Wegener's granulomatosis disease.

Waldenstroms macroglobulinemia is a type of non-Hodgkin's lymphoma that has been associated with increased monoclonal IgM produced from abnormal B lymphocytes (30), causing hyperviscosity of the blood.

Although the precise trigger for polymyositis remain unknown, distinct subsets of clinical patient sera exist that are characterized by antibodies directed against specific nuclear and cytoplasmic antigens, including histidyl tRNA synthetase (Jo-1). Anti-Jo-1 antibodies are almost completely specific for myositis, being more common in polymyositis than dermatomyositis (31).

Other Possible Antigenic Markers

Generalized myasthenia gravis (GMG) is an autoimmune disorder generally thought to be caused by an antibody-mediated attack against the skeletal muscle nicotinic acetylcholine (Ach) receptor (AchR) at the neuromuscular junction (32). Extraocular muscle weakness and double vision are present in about 90% of patients with myasthenia gravis, termed ocular myasthenia gravis (OMG). Both forms are sometimes associated with thyroid autoimmunity or thyroid-associated ophthalmopathy (TAO). The sera of patients with GMG and OMG were analyzed by Western blot analysis for antibodies against porcine eye muscle membrane proteins and by ELISA for reaction with two skeletal muscle antigens that are prominent markers for TAO, the calcium-binding protein, calsequestrin, and the flavoprotein subunit of mitochondrial succinate dehydrogenase. The most frequently found antibodies were those targeting eye muscle membrane proteins of 15, 67, and 110 kDa. Antibodies reactive with purified calsequestrin (63 kDa) were detected in 21% of patients with OMG but none with GMG (32).

Goodpasture syndrome is a life-threatening autoimmune kidney disease. In 1988, the associated autoantibodies were found to be specific for the noncollagen region of the $\alpha 3$ collagen IV chain (33), which binds to the glomerular and alveolar basement membranes, causing progressive glomerulonephritis and pulmonary hemorrhage. Two major conformational epitope regions have been identified

on the noncollagen region of the $\alpha 3$ collagen IV chain as residues 17–31 [E(A)] and 127–141 [E(B)] (34).

Scanning Systems

Coupled with the production of protein microarrays for the sensitive detection of these disease markers using ζ -grip is the visualization of the array results. Unlike most DNA microarrays that utilize fluorescent scanning (19), Miragene (Anaheim, CA) has developed a nonfluorescent protein microarray scanning system and software package that yields high-quality repeatable results and works with the ζ -grip platform described here.

RESULTS AND DISCUSSION

In order to validate the use of the ζ -grip system for disease biomarker detection, low-cost multianalyte SLE-specific autoantigen panels were developed. Figure 1 shows the results of six arrays comparing SLE patient and control sera for a range of titers. The antigens listed in Table 1 were spotted onto six ζ -grip slides, where three of the slides were developed using a pool of SLE patient serum as primary antibody (with a titer of 1:100 on one slide, 1:200 on the other slide, and 1:500 for the third). The other three slides were developed using a pool of control serums corresponding to the age and sex of the SLE patients as primary antibody, also with titers of 1:100, 1:200, and 1:500. A positive result is defined by five positive replicates per row,

Table 1. Location and Type of Antigens Spotted on the ζ -Grip Slides Featured in Figures 1–3 and 5

	A	B	C	D	E	F	G	H	I
1	Control +ve	SSA	SSA10/30	SCL5/35	SCL1/39		PCA1/39	F2A2 his	F2A210/30
2	SSA 5/35	SSA1/39	SSB	Jo-110/30	Jo-15/35	Jo-11/39	F2A25/35	F2A21/39	whole his
3	SSB10/30	SSB5/35	SSB1/39	MIT	MIT10/30	MIT5/35	whole10/30	whole5/35	whole1/39
4		SmA	SmA10/30	MIT1/39	RAG	RAG10/30			
5	SmA5/35	SmA1/39	SRC	RAG5/35	RAG1/39	Clq	H2b	H2b10/30	H2b5/35
6	SRC10/30	SRC5/35	SRC1/39	Clq10/30	Clq5/35	Clq1/39	H2b1/39	H3 & H4	H3&H410/30
7		SCL	SCL10/30	PCA	PCA10/30	PCA5/35	H3&H45/35	H3&H41/39	F4
8	SCL5/35	SCL1/39	Jo-1	PCA1/39	F2A2	F2A210/30	F410/30	F45/35	F41/39
9	Control +ve		SSA10/30	F2A25/35	F2A21/39	Whole his	Control +ve		
10	SSA 5/35	SSA1/39		Jo-110/30	Jo-15/35	Jo-11/39	whole10/30	whole5/35	whole1/39
11	SSB10/30	SSB5/35	SSB1/39		MIT10/30	MIT5/35	H2b histone	H2b10/30	H2b 5/35
12			SmA10/30	MIT1/39		RAG10/30	H2b1/39	H3&H4	H3&H410/30
13	SmA5/35	SmA1/39		RAG5/35	RAG1/39		H3&H410/30	H3&H41/39	F4
14	SRC10/30	SRC5/35	SRC1/39	Clq10/30	Clq5/35	Clq1/39	F410/30	F45/35	F41/39
15			SCL10/30		PCA10/30	PCA5/35			
16									
17									
18									
19									
20	Control +ve								

The dilutions of the antigens are provided: 10/30 indicates 10 μ L of the original antigen in 30 μ L of phosphate-buffered saline (PBS); 5/35 indicates 5 μ L of the original antigen in 35 μ L of PBS; and 1/39 indicates 1 μ L of antigen in 39 μ L of PBS. Bolded antigens indicate positive responses to disease serum used in the experiments mentioned in this article. To be considered a positive response, each box/location will contain five purple spots on the ζ -grip. Nonbolded antigens were spotted on the ζ -grip slides, but did not react with the control or disease serum (as indicated by no spots at their location). All antigens were spotted using the SpotBot robotic arrayer. Empty cells indicate PBS only was added. Control +ve are positive controls (human IgG) used for calibration.

and a negative result is defined as zero to four positive replicates per row, although false negative results may result. As can be seen, the slides using an SLE patient pool as primary antibody reacts to a number of antigens including SSA, SSB, mitochondrial antigen (MIT), Smith/ribonucleoprotein (RNP) complex (SRC), recombination activating protein (RAG), whole histones, F2A2 histones, and H2b histones, thereby showing the feasibility of system to perform a multi-analyte immunoassay. Positive reactions that appeared on the SLE patient slides but not on the control slides indicate the locations of disease markers, which can easily be traced back to the 384-wells dish and identified in Table 1. Pure SSA is positive at B1, where SSA

10/30 is positive at C1 and C9, and SSA 5/35 is positive at A2. Pure SSB is positive at C2. The SRC antigen is positive at C5, and MIT is positive at D3. The RAG is positive at E4, RAG 10/30 is positive at F4 and F12, and RAG 5/35 is positive at D5 and D13. Autoantibodies against histones are also present in SLE patients. The positive histones are whole histones at F9 and I2, F2A2 at H1, pure H2b at G12, H2b 10/30 at H12, and H2b 5/35 at I12. On the corresponding control slide, only the positive controls (human IgG) react.

ELISA testing typically uses an antibody titer of 1:100 to detect autoimmune diseases. Although weaker than the 1:100 titer, a titer of 1:500 using the present method gave a strong signal. In fact, using the ζ -grip slide we have visualized a signal up to a titer of 1:1000, and, in some cases, have shown a signal at a titer of 1:10,000 (data not shown). When corresponding ELISA titers were tested for all samples, the ζ -grip platform gave results 10–200 times more sensitive than ELISA, depending on the conditions used for the ζ -grip assay (data not shown). This comparison can also be argued based on the Ekins equilibrium model (35). Based on these results, this method appears much more sensitive than ELISA. While some spots appear darker than others, this is explained by the fact that the more autoantibodies present in the autoimmune disease sera, the stronger the signal (36). Disease markers can be detected using ζ -grip at titers higher than 1:1,000,000 when using a patient's sera.

An analogous study performed using the same antigen panel and a pool of SS patients' serum as the primary antibody provides further validation of the ζ -grip system. The results are shown in Figure 2. Antigens that respond to the SS patient serum include pure SSA at B1, where SSA 10/30 is positive at C1 and C9, and SSA 5/35 is positive at A2 and A10. Pure SSB and MIT also respond to SS and are seen at C2 and D3, respectively.

To rule out the possible generation of false positive signals (specifically from an abnormal immune system associated with the autoimmune disease), the same antigen panel was analyzed with four different pools of patient sera (as primary antibodies): (i) Waldenström's macroglobulinemia, (ii) Wegener's granulomatosis disease, (iii) Goodpasture syndrome, and (iv) myasthenia gravis. Pools of age- and sex-matched control patient serum were also prepared and used as the control primary antibodies. Only the positive control marker (secondary antibody) should react, as none of the antigens used are known to be autoantigenic for these diseases. Results are shown in Figure 3. As can be seen, neither Waldenström's nor Wegener's serum pools react to antigens on the array. The myasthenia gravis serum has four positive reactions located at the histone antigen. However, because we define a positive as a total of five positive replicates, we take this reaction to be negative. It should be noted, however, that histones are highly charged and do not interact as well with the hydrophobic substrate as other tested antigens. The Goodpasture syndrome serum, on the other

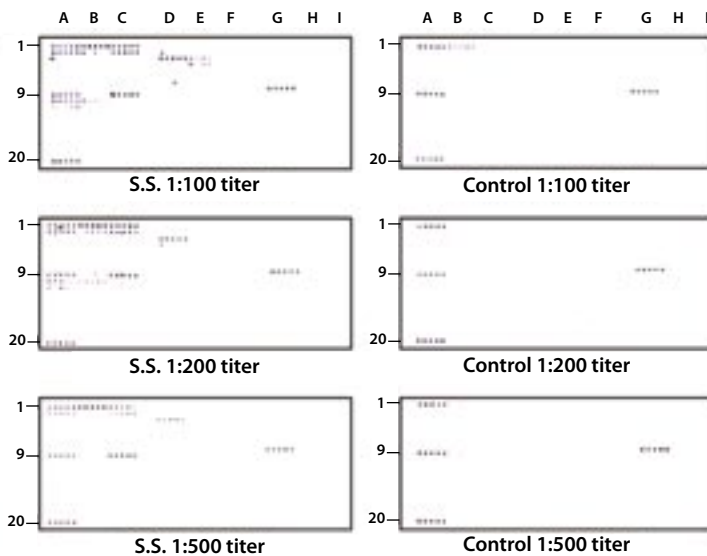


Figure 2. SS multianalyte microarray panel results. Results of six arrays using a pool of five SS patients' serum, or a pool of five corresponding age- and sex-matched control patients' serum, for a range of titers. Positive responses can be traced back as in Figure 1, and the same antigen panel as in Figure 1 was used.

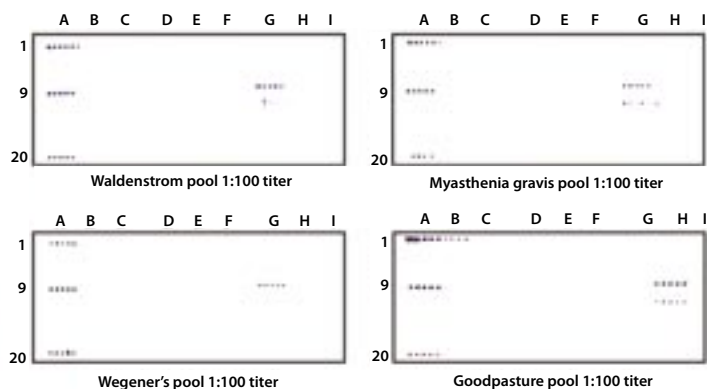


Figure 3. Cross-reactivity results. Four assays using a pool of five Waldenström patients, five Wegener patients, five myasthenia gravis patients, or five Goodpasture patients were used to determine cross-reactivity between other autoimmune diseases and antigens used.

hand, shows a five positive replicate response at both the SSA antigen and histone H2b antigen, indicating that the patient serum has an autoantibody response to both SSA and histone H2b. This phenomenon could be a false positive, but could also be due to overlapping and mixed symptoms associated with the autoimmune disease. It is possible that the Goodpasture pool might consist of a patient with Goodpasture disease, on top of being at an early stage of Sjögren's or lupus.

To determine result reproducibility, the antigens listed in Table 2 were printed every two weeks from the same 384-wells dish, with a freeze/thaw cycle between printings. Printing occurred five different times over the course of 2 months. The ζ -grip slides were then assayed using four different patient sera (also freeze/thawed between assays), including a patient positive for the MIT antigen (PMA), a patient positive for the Jo-1 antigen (PJA), a patient positive for the Smith (Sm) antigen (PSA), and a patient positive for the SSB antigen (PSSB), and four age- and sex-matched control sera. As can be seen in Figure 4A, the three positive control locations appear in both patient serum and normal serum. Positive responses are seen at A6 for PMA and at A4 for PJA. The sporadic appearance of signals at location A8 indicates a suspected contamination, which was solved by increasing the percentage of ethanol in the wash buffer, and cleaning the printer. Autoantibodies to the Sm antigen have been observed in 15%–30% of SLE sera (37) and have been described as precipitates in sera of patients with SLE (38). Additionally, these antibodies are usually accompanied by antinuclear ribonucleoprotein (nRNP) antibodies (39). Results shown in Figure 4b indicate a positive response is located at location K7 (the RNP antigen), consistent with the Sm-SLE connection previously observed (37–40). In addition, PSA has five signals at C6 (the Sm antigen). There is also positive response at location B5 (the RNP antigen). PSSB responds to both C2 [SSB antigen from BioDesign (Saco, ME)] and A7 [SSB antigen from ImmunoVision (Springdale, AR)]. The results shown here demonstrate the repeatability and stability of the assays using this system, as well as further proving the feasibility of the ζ -grip platform, as the patient serum only reacts with its corresponding antigen, and the control serum did not give false positives.

Figure 1 shows disease markers for SLE, which by comparison to SS demonstrates obvious overlap for these autoimmune diseases. A comparison between the SLE 1:100 and the SS 1:100 titers is shown in Figure 5. Here it can be seen that both SLE and SS patients respond to all dilutions of SSA located at A2, A10, B1, C1, and C9, SSB at C2, and MIT located at D3. Unique antigens to SLE are the whole histones (F9 and I2), F2A2 (H1), and H2b (G12, H12, and I12), SRC at C5, and all dilutions of RAG at E4, F4, F12, D5, and D13. In order to distinguish SS patients from SLE patients, a wider range of antigens are needed to identify unique antigens for SS.

Table 2. Location and Type of Antigens Spotted on the ζ -Grip Slides Featured in Figure 4

	A	B	C
1	Control+ve		
2			SSB(BioD)
3	SmA(BioD)	pANCA(BioD)	cANCA(BioD)
4	Jo-1(BioD)	ApoH(BioD)	Scl-70(BioD)
5	Jo-1(immuno)	RNP(immuno)	Scl-70(immuno)
6	MIT(immuno)	DNA(immuno)	SmA(immuno)
7	SSB(Immuno)	SSA(immuno)	Control+ve
8	IgA		Control+ve

Antigens purchased from BioDesign are labeled with (BioD), and antigens purchased from ImmunoVision are labeled with (immuno). Each box represents five dots of the same antigen (indicated above) spotted onto the ζ -grip slides using the Spotbot robotic arrayer. Empty cells indicate PBS only was added. Control +ve are positive controls (human IgG) used for calibration.

Unique markers for SLE include the histones, located at H1, G12, and F9 in Figure 3. This is not surprising, as it was found that the predominant responses to histones in SLE sera were to H1, H2, and H3. In fact, binding occurred to H1 and H2b in 33% of patients, while 25% showed higher binding to H3 (40). Other unique disease markers for SLE include SRC antigen and RAG. Overlapping antigens include, SSA, SSB, and MIT, as both SLE and SS autoantibodies react to these antigens. Due to the limited number of antigens at hand, unique disease markers for SS are not included in our array. However, possible markers for SS may include saliva IgA autoantibodies against muscarinic acetylcholine receptors (mAChRs) (41), and the thyroid hormone autoantibody (THAb) (42).

While other protein arrays have been described (20), the ζ -grip system described has the following advantages. Glass arrays do not allow the extent of protein binding or a better configuration for epitope analysis. Silanated glass slides (lysine, epoxy, aldehyde, etc.) are designed for nucleotide linearization and bind too aggressively to proteins and require extreme pH conditions (i.e., 9.5), which results in low immunosensitivity (presumably because epitopes are destroyed in such an adverse environment).

Our system is better than hydrophilic surfaces, like nitrocellulose and gels, because samples can migrate in these polymers. This diffusion can result in large uneven spots that are hard to quantify and have less protein density.

The ζ -grip system is more comparable to ELISA, but it is more sensitive, can be multiplexed, and is lower in cost. A two-color fluorescent system is not equivalent to ELISA (not enzyme-linked) and is not sensitive enough to provide a good signal-to-noise ratio for proteins, as there is no PCR amplifica-

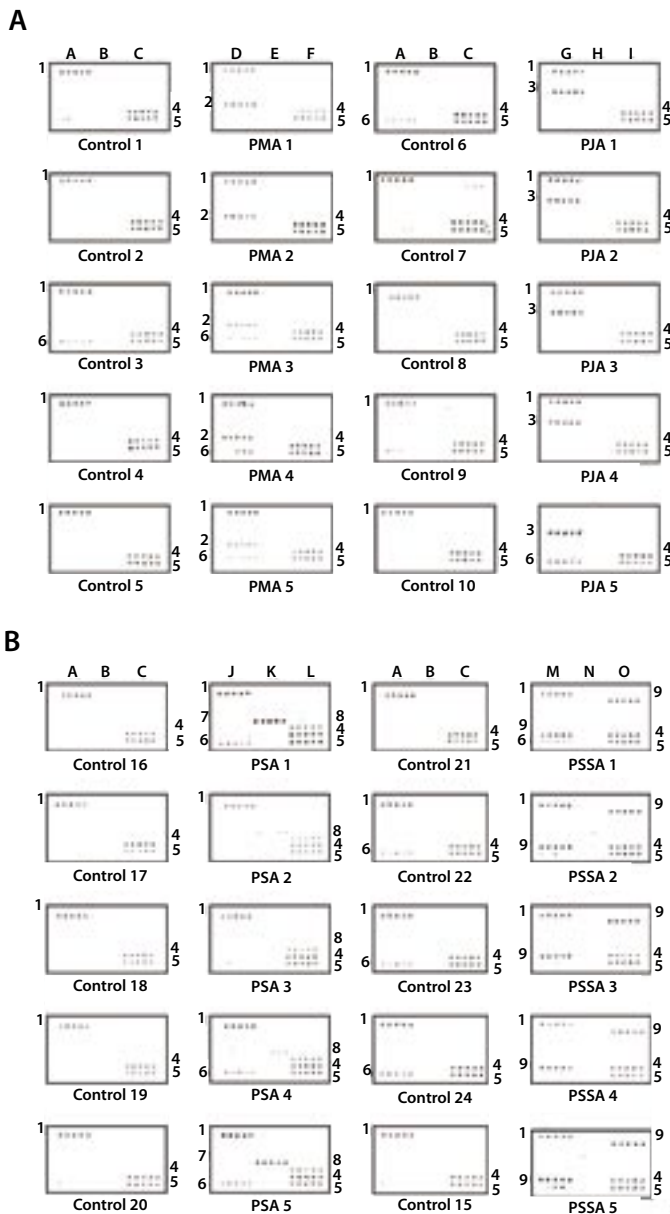


Figure 4. Stability results from the same set of antigens printed on 40 ζ -grip slides over a period of 2 months. All antigens and human sera were freeze/thawed for each run, where tests were repeated five times, in which positive responses were traced back to the antigens in the 384-well dish, identified in Table 2. The different rows are independent replicates performed every 2 weeks. Four human serums were used, including patients having antibodies against (A) MIT or Jo-1 or (B) Sm or SSB. Control slides 1 to 10 are the normal human serum that acts as a negative control for the experiment.

tion step to ensure an abundance of target. Proteins tend to be more “fickle” as they not only need to be present but must also have measurable activity.

We have demonstrated that the ζ -grip protein microarray system is a feasible, new, and capable method for performing multianalyte immunoassays. Also, the system allows multiple analysis and internal repeats (as each chip holds 20,000 antigens). In the newly developed format, the system can accommodate titrations of 10 different sera and/or dilutions with 1000 antigens each, where each well utilizes 10 μ L of diluted patient serum, consistent with trends in molecular medicine, as it can handle a large number of disease markers. Currently, this system is being used to identify new autoimmune disease markers (manuscript in preparation).

MATERIALS AND METHODS

Antigens and Serum

SSA antigen, SSB antigen, Sm, myeloperoxidase antigen (pANCA), proteinase 3 antigen (cANCA), Jo-1, apolipoprotein H (ApoH), DNA topoisomerase (Scl-70), Sm/RNP, RAG, PCA, chloroquine (Clq), SRC, MIT whole histone, His F2b, His F2a2, His F3, and F2a1 were obtained from ImmunoVision. All of the patient and control serum were purchased from ProMedDx, LLC (Norton, MA). Other sera purchased from ProMedDx, LLC include pools of Waldenstrom’s, Wegener’s, Goodpasture, and myasthenia gravis disease (five patients for each pool).

Substrates

The ζ -grip slides described and used here are composed of a porous hydrophobic membrane with a 3-dimensional binding structure. A protein’s internal hydrophobic bonds, which maintain its secondary and tertiary structure, are assumed to partially exchange for protein- ζ -grip hydrogen bonds and result in hydrophilic exterior epitopes that are displayed for antibody binding. Such binding is irreversible except when a detergent is used (i.e., Tween[®]20). In addition, the hydrophobic nature of the chip results in small, “tall” (90° contact angle) microarray spots that do not diffuse through the substrate. These characteristics affect epitope presentation by allowing dense, round spots that are of critical importance for the high signal-to-noise ratio observed.

Procedure

In general, the procedure to measure human disease marker is to first spot antigens (1-nL volume) from a 384-well dish onto the ζ -grip slide, using an array printer. The antigens were spotted based on equivalent activity values (determined using ELISA and World Health Organization standards) and in general, the mass range was between 0.01–1 pg/nL. A set of spots is included on each array for normalization and is predetermined to be just below saturation. The normaliza-

tion of spots corrects for chip-to-chip differences and maintains signal in a quantifiable region (if unknown signal is equal to or greater than the normalization signal, it is considered oversaturated and not quantifiable). The dynamic range of the assay is 3 orders of magnitude. When compared to fluorescence (which appears to be approximately 5 orders of magnitude), our assay is more sensitive. These studies were analyzed by statistical analysis of saturated and minimal detected signals (data not shown). The use of the array printer allows for simultaneous and repeated analysis of various chemistries onto the ζ -grip. Once slides have been developed, they can be scanned using the Miragene scanning system, where the resulting image is then quantified using commercial microarray software, such as ArrayVision™, Molecularware™, or TIGR™ (43–45).

Spotting Antigens

Antigens were spotted as indicated previously onto ζ -grip slides using the SpotBot® Personal Microarray Robot (Miragene/TeleChem International, Inc.). Each target antigen was aliquoted into individual wells of the 384-well dish (Phenix Research Products, Hayward, CA) at a minimal volume of 5 μ L/well. The dish was then placed on the left side of the SpotBot, and 12 ζ -grip slides were fitted on the right side of the instrument (with two plain microscope slides in the preprint area). The SpotBot software program used was SPOCLE (SpotBot Control Language) Generator, and the settings used were: Factory Default Profile; pintype SMP3; pin configuration set to 1 \times 1; partial microplate; total microplate count set to 1; and spots per sample set to 5. The settings in microarray printing were: spot spacing set to 300 μ m; subgrid dimension set to 15 \times 15 (column \times row); print offset set to 3.0 \times 5.0 (lateral \times vertical); cleaning cycle set to 10 washes; and the rest of the settings kept as computer default. See SpotBot protocol in Reference 46. Tables 1 and 2 map the location of the antigens in the 384-well dish for the experiments later described.

DEVELOPMENT OF ζ -GRIP SLIDES

All printed ζ -grip slides were labeled and individually placed into sterile plastic dishes, prefilled with proprietary blocker. Loaded dishes were then placed on an ELISA shaker (Titer Plate Shaker; LabLine Instruments, Melrose Park, IL) and rotated (to allow for continuous mixing) at room temperature for 1 h. Next, diluted patient serum at desired titer (known generally as primary antibody) was added to each dish (with the slide and blocker) and continuously mixed for 1 h. Solutions were appropriately discarded, and slides were washed three times by adding 10 mL of wash buffer. After the last wash, 10 mL of proprietary detector was added. This reagent is catalyzed by alkaline phosphatase and further developed as in a standard ELISA. The dishes were shaken for 1 h at room temperature. Solutions were discarded from the dishes, and slides

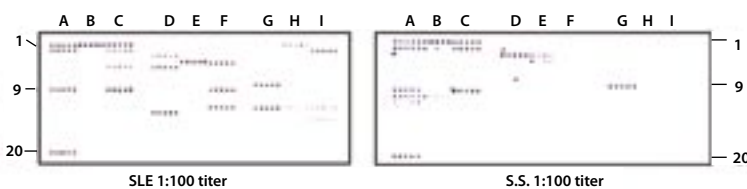


Figure 5. Comparison of two arrays using SLE and SS patients. Positive responses can be traced back to the original antigen in the 384-well dish identified in Table 1.

were again washed three times. After discarding the last wash, 10 mL of developer was added to the dish and shaken at room temperature for 15 min. The developer was discarded, and tap water was added to the dishes for 2 min to stop further development, after which ζ -grip slides were left to air-dry overnight.

Scanning and Analysis

Dried slides were placed face down onto a commercial flatbed scanner (Miragene). The scanner consists of a plastic template (with openings to hold 1–20 ζ -grip slides) secured onto the scanner, to allow the relative positions of the ζ -grips to remain constant during scanning. The scanner mode was set to about 1600 dpi (dots per inch) to get a clear, high-quality image. The previewing mode was first chosen prior to scanning, so that specific areas containing the microarray spots could be isolated. Once the desired area was selected, the slides were scanned and analyzed using the appropriate software (such as ArrayVision, TIGR, and MolecularWare). The software allows for rapid and automated analysis of the resulting protein array images (37–39). Most other work has been done using fluorescent scanners, although analyte binding to microarrays using gold nanoparticle labels and a desktop scanner was recently described (47).

COMPETING INTERESTS STATEMENT

Dr. S. Lebrun declares that, as the Director of Research and Development at Miragene, Inc., he invented the substrate, chemistry, and imaging system described in this article and receives royalties from the resulting sales.

REFERENCES

1. **Kuby, J.** 1997. Immunology, 3rd ed. W.H. Freeman and Company, New York.
2. **Bora, U., L. Chugh, and P. Nahar.** 2002. Covalent immobilization of proteins onto photoactivated polystyrene microtiter plates for enzyme-linked immunosorbent assay procedures. *J. Immunol. Methods* 268:171.
3. **Pandley, A. and M. Mann.** 2000. Proteomics to study genes and genomes. *Nature* 405:837-846.
4. **Lebrun, S.J.** 1997. Ethanol shifts the DNA damage response from error-free DNA repair to a mutagenic error prone pathway. Dissertation. University of California, Irvine.
5. **Bartel, P.S. and S. Fields.** 1995. Analyzing protein-protein interactions using the two-hybrid system. *Methods Enzymol.* 254:241-263.

6. **Phizicky, E.M. and S. Fields.** 1995. Protein-protein interactions: methods for detection and analysis. *Microbial Review* 59:94-123.
7. **Lebrun, S.J., R.L. Shpall, and L. Naumovski.** 1998. Interferon-induced upregulation and cytoplasmic localization of Myc-interacting protein Nmi. *J. Interferon Cytokine Res.* 18:767-771.
8. **Formosa, T. and B.M. Alberts.** 1984. The use of affinity chromatography to study proteins involved in bacteriophage T4 genetic recombination. *Cold Spring Harbor Symp. Quant. Biol.* 49:363-370.
9. <http://www.immunovision.com/pg0007.htm>. 10/26/2002.
10. <http://www.chemicon.com/Resource/ANT101/a2C.asp?TXTSEA RCH=troubleshooting> 10/24/2002.
11. **Kodadek, T.** 2001. Protein microarrays: prospects and problems. *Chem. Biol.* 8:105-115.
12. **Kodadek, T.** 2002. Development of protein-detecting microarrays and related devices. *Trends Biochem. Sci.* 27:295-300.
13. **Arenkov, P., A. Kukhtin, A. Gemmell, S. Voloshchuk, V. Chupeeva, and A. Mirzabekov.** 2000. Protein microchips: use for immunoassay and enzymatic reactions. *Anal. Biochem.* 278:123-131.
14. **Macbeath, G. and S.L. Schreiber.** 2000. Betting on tomorrow's chips. *Science* 289:1760-1763.
15. **Englert, C.R., G.V. Baibakov, and B.M. Emmert.** 2000. Layered expression scanning: rapid molecular profiling of tumor samples. *Cancer Res.* 60:1526-1530.
17. **Stillman, B.A. and J.L. Tonkinson.** 2000. FAST slides: a novel surface for microarrays. *BioTechniques* 29:630-635.
18. <http://lifesciences.perinellmer.com/downloads/P10482.pdf> 10/26/2002.
19. **Schena, M.** 2000. *Microarray Biochip Technology*, p. 28. Eaton Publishing, Natick.
20. **Mehta, M.M., R. Munker, B. Kranz, E. Thiel, and S. Thierfelder.** 1983. Immunofluorescence with monoclonal antibodies on poly-L-lysine coated slides: an alternative to conventional methods. *Blut.* 47:237-242.
21. **Robinson, W.H., C. DiGennaro, W. Hueber, B.B. Haab, M. Kamachi, E.J. Dean, et al.** 2002. Autoantigen microarrays for multiplex characterization of autoantibody responses. *Nat. Med.* 8:295-301.
22. **Billaut-Mulor, O., C. Cocude, V. Kolesnitchenko, M.J. Truong, E.K. Chan, E. Hachula, et al.** 2001. SS-56, a novel cellular target of autoantibody responses in Sjögren syndrome and systemic lupus erythematosus. *J. Clin. Invest.* 108:861-869.
23. **Mamula, M.J., O.F. Fox, H. Yamagata, and J.B. Harley.** 1986. The Ro/SSA autoantigen as an immunogen some anti-Ro/SSA antibody bind IgG. *J. Exp. Med.* 164:1889-1901.
24. **Rader, M.D., C. O'Brien, Y.S. Liu, J.B. Harley, and M. Reichlin.** 1989. Heterogeneity of the Ro/SSA antigen, different molecular forms in lymphocytes and red blood cells. *J. Clin. Invest.* 83:1293-1298.
25. **Sutton, I. and J. Neuberger.** 2002. Primary biliary cirrhosis: seeking the silent partner of autoimmunity. *Gut* 50:743-744.
26. **Strassburg, C.P., E. Jaeckel, and M.P. Manns.** 1999. Anti-mitochondrial antibodies and other immunological tests in primary biliary cirrhosis. *Eur. J. Gastroenterol. Hepatol.* 11:595-601.
27. **Quinn, J., A.G. Diamond, J.M. Palmer, M.F. Bassendine, O.F. James, and S.J. Yeaman.** 1993. Lipoylated and unlipooylated domains of human PDC-E2 as autoantigens in primary biliary cirrhosis: significance of lipoate attachment. *Hepatology* 18:1384-1391.
28. **Metcalf, J.V., H.C. Mitchison, J.M. Palmer, D.E. Jones, M.F. Bassendine, and O.F. James.** 1996. Natural history of early primary biliary cirrhosis. *Lancet* 348:1399-1402.
29. **Escalona, M., F.J. Lopez-Longo, C.M. Gonzalez, I. Monteagudo, M. Rodriguez-Mahou, R. Grau, and L. Carreno.** 2002. Anti-SA sera from patients with rheumatoid arthritis contain at least two different subpopulations of anti-SA antibodies. *J. Rheumatol.* 29:2053-2060.
30. **Van der Woude, F.J., N. Rasmussen, S. Lobatto, A. Wiik, H. Permin, L.A. van Es, et al.** 1985. Autoantibodies against neutrophils and monocytes: tool for diagnosis and marker of disease activity in Wegener's granulomatosis. *Lancet* 1:425-429.
31. **Hainsworth, J.D.** 2000. Monoclonal antibody therapy in lymphoid malignancies. *Oncologist* 5:376-384.
32. **Reichlin, M., P.J. Maddison, I. Targoff, T. Bunch, F. Arnett, G. Sharp, et al.** 1984. Antibodies to a nuclear/nucleolar antigen in patients with polymyositis overlap syndrome. *J. Clin. Immunol.* 4:40-44.
33. **Gunji, K., C. Skolnick, T. Bednarczuk, S. Benes, B.A. Ackrell, B. Cochran, et al.** 1998. Eye muscle antibodies in patients with ocular myasthenia gravis: possible mechanism for eye muscle inflammation in acetylcholine-receptor antibody-negative patients. *Clin. Immunol. Immunopathol.* 87:276-281.
34. **Jaskowski, T.D., et al.** 2002. Comparison of four enzyme immunoassays for the detection of immunoglobulin G antibody against glomerular basement membrane. *J. Clin. Lab. Anal.* 16:143-145.
35. **Borza, D.B., K.O. Netzer, A. Leinonen, P. Todd, J. Cervera, J. Saus, and B.G. Hudson.** 2000. The goodpasture autoantigen. Identification of multiple cryptic epitopes on the nc1 domain of the alpha3(iv) collagen chain. *J. Biol. Chem.* 275:6030-6037.
36. **Ekins, R.P.** 1989. Multi-analyte immunoassay. *J. Pharm. Biomed. Anal.* 7:155-168.
37. **Joos, T.O., M. Schrenk, P. Hopfl, K. Kroger, U. Chowdhury, D. Stoll, et al.** 2002. A microarray enzyme-linked immunosorbent assay for autoimmune diagnostics. *Electrophoresis* 23:2641-2650.
38. **Tan, E.M.** 1982. Special antibodies for the study of systemic lupus erythematosus. *Arthritis Rheum.* 25:753-756.
39. **Tan, E.M. and H.G. Kunkel.** 1966. Characteristics of a soluble nuclear antigen precipitating with sera of patients with systemic lupus erythematosus. *J. Immunol.* 96:464-471.
40. **Yasuma, M., Y. Takasaki, K. Matsumoto, A. Kodama, H. Hashimoto, and S. Hirose.** 1990. Clinical significance of IgG anti-Sm antibodies in patients with systemic lupus erythematosus. *J. Rheumatol.* 17:469-475.
41. **Elgin, S.C.R. and H. Weinbrauh.** 1975. In E.E. Smell, P.D. Boyer, A. Meister, and C.C. Richardson (Eds.), *Chromosomal Proteins and Chromatin Structure in Annual Review of Biochemistry*, p. 725. Annual Reviews, Palo Alto.
42. **Berra, A., L. Sterin-Borda, S. Bacman, and E. Borda.** 2002. Role of salivary IgA in the pathogenesis of Sjogren syndrome. *Clin Immunol.* 104:49-57.
43. **Ruggeri, R.M., M. Galletti, M.G. Mandolino, P. Aragona, S. Bartolone, G. Giorgianni, et al.** 2002. Thyroid hormone autoantibodies in primary Sjogren syndrome and rheumatoid arthritis are more prevalent than in autoimmune thyroid disease, becoming progressively more frequent in these diseases. *J. Endocrinol. Invest.* 25:447-454.
44. <http://www.imagingresearch.com/products/ARV.asp>
45. <http://www.molecularware.com>
46. <http://www.tigr.org/>
47. **Telechem International.** 2001. *ArrayIt™-Spotbot™ Personal Microarray Robot Handbook*. Telechem International, Inc., Sunnyvale, CA.
48. **Han, A., M. Dufva, E. Belleville, and C.B. Christensen.** 2003. Detection of analyte binding to microarrays using gold nanoparticle labels and a desktop scanner. *Lab Chip* 3:329-332.

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