

Protein Arrays for Serodiagnosis of Disease

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Summary

Protein microarrays offer the possibility to circumvent most of the current limitations in the serodiagnosis of allergy, autoimmune, and infectious disease by allowing the simultaneous, multiparametric determination of specific subclasses of antibodies directed against many pathogenic antigens. Microarray immunoassays have been developed with these characteristics. A first-generation assay, for the serodiagnosis of infectious disease, allows the determination of IgG and IgM antibodies to various viral and bacterial antigens. In addition, a second-generation assay, designed for the serodiagnosis of allergic disease, permits the determination of IgE antibodies to various allergens implicated in allergic disease. Slides printed with antibody dilution curves and antigen are first incubated with serum samples and then subsequently with secondary antibodies. For detection of human IgG and IgM, fluorescently labeled secondary antibodies are employed. However, because of low-level concentrations of circulating IgE antibodies, a more sensitive protocol is required for human IgE detection. Here, fluorescence is delivered via the coupling of the secondary antibody to tyramide signal-amplification reagent. Human IgG, IgM, or IgE bound to the printed antigens can then be revealed by confocal scanning microscopy and quantified with internal calibration curves. Generation of analytical and clinical data have demonstrated that the microarray test format provides equivalent performance to enzyme-linked immunosorbent assay (ELISA) tests and offers a significant advantage in convenience and cost when compared to traditional test formats.

Key Words:

Protein microarray; immunoassay; signal amplification; serodiagnosis; allergy; infectious diseases.

1. Introduction

In vitro immunoassays form the basis of many clinical diagnostic tests. Assays capable of antigen-specific antibody detection, in the form of ELISA-based tests (*1*), are widely available and are routinely employed in the clinical laboratory. However, certain characteristics of these assays, notably large sample/reagent volume require-

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ments, procedure-intensive protocols, and the absence of true parallelism, render them unsuitable for clinical applications that require the ability to determine numerous parameters in a timely and cost-effective manner (2,3). With the establishment of robust, automated microdeposition technology allowing the construction of high-density ordered arrays of molecules (microarrays) (4), the capability now exists that, in overcoming most of the current ELISA limitations (5), could support a truly parallel, multiparametric, immunoassay platform. Such miniaturized microspot ligand-binding assays were first described over a decade ago by Ekins et al. in the "ambient analyte theory," which demonstrated the feasibility of miniaturizing standard ligand-binding chemistry and predicted superior sensitivity for this assay format over others (6–8). The realization of miniaturized immunoassays, however, has been held back by the absence of the necessary instrumentation that is essential for their development and analysis. Driven by interest in high-density nucleic acid arrays for gene-expression studies, these tools have now become available as standard laboratory equipment and can be used for the generation of protein microarrays (9). Microdeposition technology, fluorescent scanners, and associated software are in place, enabling image generation and signal quantification from printed arrays of proteins. Early progress in protein arraying has seen the development of comparative fluorescence assays for measuring the concentrations of several specific proteins and antibodies in complex solutions (10–12). Furthermore, remarkable advances have been made in the development of antigen microarrays for the detection of specific antibodies in clinical samples (13–16). Results to date show that the microarray test format provides equivalent performance to ELISA (ref. 13 and Table 1), indicating that microarray tests can be incorporated in a fully automated random-access immunoassay platform for routine use in clinical chemistry laboratories.

Here, the authors document methods for the construction and analysis of two distinct microarray-based immunoassays. The first, tailored to the serodiagnosis of infectious disease, permits the simultaneous, quantitative determination of IgG and IgM antibodies to the ToRCH panel (*Toxoplasma gondii*, rubella virus, cytomegalovirus [CMV], and herpes simplex virus [HSV] types 1 and 2) in human serum. The second, designed for the serodiagnosis of allergic disease, permits the simultaneous, quantitative determination of serum IgE antibodies to various common allergens.

2. Materials

2.1. Equipment

1. Computer-controlled high-speed robot (Total Array System; Biorobotics).
2. S5000 scanner including ScanArray™ and QuantArray™ software (PerkinElmer).
3. Equipment for ELISA:
 - (i) Microtiter plate washer: Autura plate washer (Mikura).
 - (ii) Microtiter plate reader: Kinetic microplate reader, v max (Molecular Devices).
4. Spectrophotometer: DU 640 (Beckman Coulter).

2.2. Common Reagents and Materials

1. Silanized glass microscope slides (CEL Associates).
2. 96- or 384-well microtiter plates (Thermo Life Sciences).

3. Gene frames. 65 μ L (1.5 $\times$ 1.6 cm) (Abgene).
4. Signal control: Alexa 546 dye (Molecular Probes, product no A—10237) in 2X phosphate-buffered saline (PBS) containing 0.1 mL/L Tween-20.
5. Negative control: 10 g/L bovine serum albumin (BSA) in 2X PBS containing 0.1 mL/L Tween-20.

2.3. Buffers

1. PBS 1X: 0.2 g/L KCl, 1.44 g/L Na₂HPO₄, 0.24 g/L KH₂PO₄, 8 g/L NaCl, pH 7.4.
2. Serum sample, horseradish peroxidase (HRP)-streptavidin conjugate and conjugated antibody diluent: 2X PBS containing 10 g/L BSA and 0.1 mL/L Tween-20.
3. Slide wash buffer: 1X PBS with 0.1 mL/L Tween-20.
4. Print buffers (see Note 1) for IgG/IgM and microbial antigens: 1X PBS containing 0.1 mL/L Tween-20 and polyvinylpyrrolidone (PVP, 10 g/L) (for human IgG and HSV1 antigen); sodium dodecyl sulphate (SDS, 0.1 g/L) (for human IgM); sucrose (100 g/L) (for CMV antigen).
5. Human IgE print buffer: 1X PBS containing 0.1 mL/L Tween-20 and 1 g/L SDS.
6. Slide blocking solution: 20 g/L BSA in 1X PBS.

2.4. Determination of Serum IgG/IgM

1. Succinimidyl Alexa 546/Alexa 594 fluorophores (Molecular Probes).
2. Microbial preparations: *T. gondii*, CMV, HSV types 1 and 2 (Radim); rubella virus antigen (grade K1S) (Microbix Biosystems).
3. Human IgG and IgM (reagent grade) (Sigma).
4. Human sera (BioMedical Resources).
5. Anti-human IgG monoclonal antibody (Radim).
6. Goat anti-human IgM antibody (OEM Concepts).

2.5. Determination of Serum IgE

1. Allergen extracts: *Dermatophagoides pteronyssinus* (D1), *Dermatophagoides farinae* (D2), *Alternaria alternata* (M6), *Olea europaea* (T9), *Artemisia vulgaris* (W6), *Dactylis glomerata* (G3), and house dust (H2) (Allergon).
2. Human IgE purified from myeloma plasma (Calbiochem).
3. Human sera (Radim).
4. HRP-streptavidin conjugate (0.1 mg/mL), Alexa 546-tyramide conjugate, amplification buffer, and 300 mL/L hydrogen peroxide as provided in the TSA™ kit no. 23 (Molecular Probes).
5. Biotinylated anti-human IgE at a final concentration of 1 mg/mL (KPL).

3. Methods

The methods detailed below describe (1) the silanization of glass slides, (2) the preparation of proteins and antibodies for printing, (3) the printing of proteins and antibodies onto silanized slides, (4) the processing of arrayed slides for IgG/IgM determination, (5) the processing of arrayed slides for IgE determination, (6) the scanning of processed slides, and (7) the quantitative analysis of the image data.

3.1. Silanization of Glass Slides

Commercial aminosilane-coated slides provided by Cel Associates are routinely used in the authors' laboratory. Some commonly used silanization methods have been documented (17).

3.2. Preparation of Proteins and Antibodies for Printing

The arrays employed in the authors' investigations are usually designed as 7×7 matrices. This format incorporates IgG and IgM antibodies and microbial antigens, that is, the ToRCH panel (see Fig. 1A), or alternatively IgE antibodies and allergens (see Fig. 2A). The respective antibodies are printed at known concentrations and serve as internal calibration curves. The panels also feature signal and negative controls.

3.2.1. Preparation of Human IgG and IgM for Printing

1. Human IgG, as provided by Sigma, is in the form of a salt-free lyophilized powder and can be stored at $2-8^{\circ}\text{C}$. Reconstitution is in 8.7 g/L NaCl to a final concentration of 1 mg/mL, and the protein concentration is determined after reconstitution by absorbance at 280 nm.
2. Once reconstituted, the IgG is aliquoted and stored at -20°C . Repeated freezing and thawing is not recommended.
3. Human IgM is supplied by Sigma as a 10 mg aliquot in 6.1 g/L Tris-HCl, 11.7 g/L NaCl, pH 8.0, containing 0.9 g/L sodium azide as a preservative. As for the IgG, the protein concentration is determined by absorbance at 280 nm. This solution is stored at $2-8^{\circ}\text{C}$. If turbidity should occur on prolonged storage, the solution can be clarified by means of centrifugation before use.
4. The IgG calibration curve (2–50 $\mu\text{g/mL}$) is prepared by diluting the human IgG preparation in 1X PBS containing Tween-20 (0.1 mL/L) and PVP (10 g/L).
5. The IgM calibration curve (0.4–8 $\mu\text{g/mL}$) is prepared by diluting the human IgM preparation in 1X PBS containing Tween-20 (0.1 mL/L) and SDS (0.1 g/L). The authors' recommendation is that the human IgG and IgM antibodies not be reused once diluted in their respective printing buffers.

3.2.2. Preparation of Microbial Antigens

All the ToRCH microbial antigens are initially diluted in 1X PBS containing Tween-20 (0.1 mL/L) and printed at a concentration of 50 $\mu\text{g/mL}$. In addition, PVP (10 g/L) is included in the HSV1 antigen solution and sucrose (100 g/L) in the CMV antigen solution. All microbial antigens are stored as aliquots at -80°C .

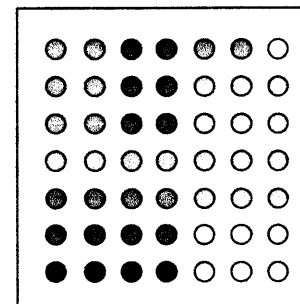
3.2.3. Preparation of Controls for the IgG/IgM Microarray

A solution of 10 g/L BSA in 2X PBS containing 0.1 mL/L Tween-20 is employed as a negative control. A solution of Alexa 546 dye (Molecular Probes) in the signal-control buffer is employed as a signal control.

3.2.4. Preparation of Human IgE

1. Human IgE provided by Calbiochem is diluted in 1X PBS to a concentration of 100 $\mu\text{g/mL}$ and stored at -20°C , according to the manufacturer's instructions. The resulting solution should not undergo freeze-thaw cycles. To avoid this problem, the solution is aliquoted prior to storage.
2. The IgE calibration curve (2–200 ng/mL) is prepared by diluting the human IgE preparation in 1X PBS containing Tween-20 (0.1 mL/L) and SDS (1 g/L). As with the human IgG and IgM antibodies, the human IgE should not be reused once diluted in its printing buffer.

A Array



Curve (pg)

- 2 IgG
 ● 10
 ● 25
 ● 50
- 0.4 IgM
 ● 2
 ● 4
 ● 8

Antigens (50pg)

- T. gondii ○
- Rubella virus ○
- CMV ○
- HSV2 ●
- HSV1 ○
- Myosin ○
- Fluorophores ○

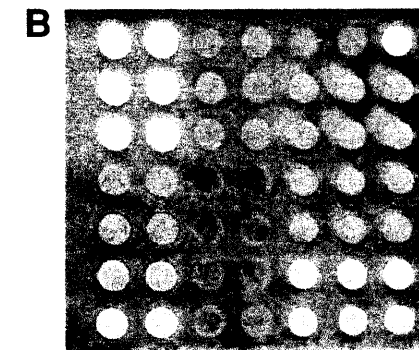


Fig. 1. Schematic array format and fluorescent scan for the microbial array. (A) Schematic representation of the microbial array format. (B) Fluorescent scan of microbial array incubated with serum and developed with Alexa 546-labelled secondary antibody.

3.2.5. Preparation of Allergens

1. Allergens are obtained as 1-g samples and are reconstituted in 1X PBS, pH 7.2, to a concentration of 10 mg/mL and stirred overnight at room temperature.
2. After centrifugation at 503g for 30 min at 10°C , the allergen extracts are filtered in four stages: first through Whatman paper (125-mm diameter, cat. no. 1001125), then through 5 μm filter disks (Pall Corp. cat. no. 4199), then through 0.45 μm filter units (Schleicher & Schuell, cat. no. 10462 450), and finally through 0.2 μm filter units (Schleicher & Schuell, cat. no. 10462 200).
3. Extracts are stored frozen at -20°C as 1.5-mL aliquots.

3.2.6. Preparation of Controls for the IgE Microarray

A solution of 10 g/L BSA in 2X PBS containing 0.1 mL/L Tween-20 is employed as a negative control. A solution of Alexa 546 dye (Molecular Probes) in the signal-control buffer is employed as a signal control. This choice of fluorophore is based on the fact that the signal-amplification technique used to process the slides makes use of the same fluorophore coupled to the tyramide.

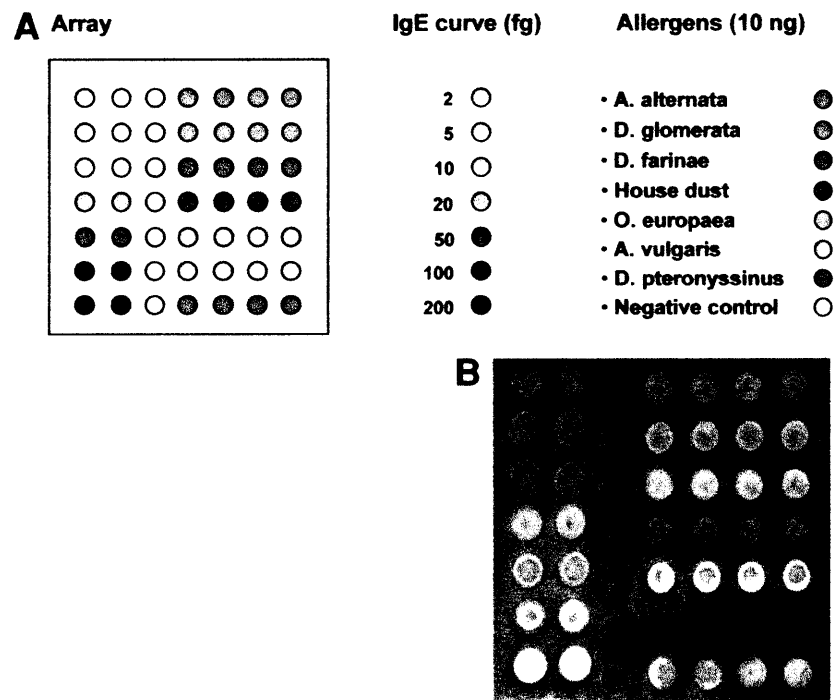


Fig. 2. Schematic array format and fluorescent scan for the allergen array. (A) Schematic representation of the allergen array format. (B) Fluorescent scan of allergen array incubated with serum and developed using the tyramide amplification protocol.

3.3. Printing of Proteins and Antibodies onto Silanized Slides

3.3.1. Preparation of the Source Plate

All the solutions for printing (antibodies/antigens/allergens) are transferred into a microtiter plate (96- or 384-well). In the authors' laboratory, 384-well plates are commonly employed for this purpose. Depending on the number of replicate arrays required, a corresponding number of pins are employed and the appropriate number of wells filled with each printing solution. At least 50 μ L of solution is added to each well. The solutions are prepared and transferred into the source plate just before the start of the print run.

3.3.2. Printing

The aforementioned antibodies, antigens, or allergens are contact printed on silanized glass slides by means of computer-controlled high-speed robotics. The samples are transferred from 384-well microtiter plates to glass slides by use of

Table 1
Comparison of Microarray and ELISA for Serum Reactivity

Allergens	ELISA		Microarray		Sensitivity %	Specificity %
	Positive	Negative	Positive	Negative		
<i>D. pteronyssinus</i>	11/22	11/22	13/22	9/22	90.9	72.7
<i>D. farinae</i>	8/16	8/16	9/16	7/16	87.5	75
<i>O. europaea</i>	7/22	15/22	8/22	14/22	71.4	80
<i>A. alternata</i>	3/20	17/20	4/20	16/20	100	94.1
<i>A. vulgaris</i>	2/22	20/22	2/22	20/22	50	95
<i>D. glomerata</i>	6/22	16/22	7/22	15/22	83.3	87.5

ELISA, enzyme-linked immunosorbent assay.

The clinical sensitivity is calculated by expressing the number of samples that are positive by microarray immunoassay as a percent of the number of samples positive for the disease (i.e., positive by ELISA).

The clinical specificity is calculated by expressing the number of samples that are negative by the microarray immunoassay as a percent of the number of samples negative for the disease (i.e., negative by ELISA).

As an example, for *Dactylis glomerata*:

	Test positive	Test negative
Disease present: 6	5	1
Disease absent: 16	2	14
Therefore:		
Sensitivity = $5/6 = 83.3\%$		
Specificity = $14/16 = 87.5\%$		

stainless steel solid pins (200 μ m diameter). Each pin is estimated to transfer approx 1 nL of sample to the slide and produces spots with a pitch of 0.6 mm. After deposition of each print solution, pins are washed first with dH₂O, then with 70% ethanol, and finally dried.

3.3.3. Storage of Printed Slides

When the printing process has been completed, the slides remain inside the robot printer cabinet for at least 12 h. This stable and controlled environment (approx 55% humidity and 25°C) is conducive to the binding of the antibodies and the antigens/allergens to the slide surface. Printed slides are stored in the dark in boxes containing silica gel bags. It is critical that the slides are kept dry; slides that have been exposed to moisture on storage give imprecise and varying signals.

3.4. Processing of Microarrayed Slides for the Determination of IgG/IgM in Human Serum

3.4.1. Preliminary Procedures

3.4.1.1. SERUM SAMPLES

Human sera are first analyzed for their reactivity against *T. gondii*, rubella virus, HSV1, HSV2, and CMV antigens by means of ELISAs (e.g., Radim EIA Well). The assays are performed according to the manufacturer's instructions. Serum samples are diluted 1:200 in serum sample diluent (see **Subheading 2.3.**) for determination of antibody reactivity to the ToRCH antigens. Samples are categorized as positive, negative, or equivocal according to the manufacturer's instructions.

3.4.1.2. FLUORESCENT LABELING OF ANTIBODIES (SEE NOTE 2)

Antibodies are labeled with the fluorophore of choice using a protein-labeling kit, for example, Alexa Fluor 546 protein-labeling kit (Molecular Probes). The efficiency of the labeling process is determined by measuring the molar ratio of dye incorporated in the protein.

3.4.1.3. PREPARATION OF THE SLIDES FOR PROCESSING

The first step in the processing of slides is the sticking of an adhesive frame (Gene-Frame) around the printed array. This frame serves two purposes: first, samples and reagents are contained within the array area; second, the frame limits the developing area to where the array is printed, thereby minimizing reagent use.

To block nonspecific binding to the activated slide surface, the printed slides are incubated for 60 min at room temperature with the slide blocking solution (see **Subheading 2.3.**). Staining jars with at least 50 mL of blocking solution are commonly used for this purpose.

3.4.2. Processing of Slides

After blocking and gentle rinsing with wash buffer (five times 1 mL) (see **Subheading 2.3.**), sera (150 μ L) are pipetted and incubated on the arrayed slides for 15 min at room temperature in a humid chamber. The use of humid chambers ensures that evaporation of samples/reagents is kept to a minimum. After the serum incubation, slides are rinsed with wash buffer (five times 1 mL).

To reveal the IgG and IgM bound to the printed antigens, two different protocols are followed:

- (i) IgG bound to the printed antigens are detected by incubating the slides for 5 min at room temperature with Alexa 546-labeled anti-human IgG (150 μ L) monoclonal antibody, diluted in the appropriate buffer.
- (ii) IgM bound to the printed antigens are revealed by incubating the slides for 5 min at room temperature with Alexa 594-labeled goat immunoglobulins (150 μ L) directed against the human IgM μ chain. It is possible to perform both processes simultaneously by incubating with a mixture of an anti-IgG and an anti-IgM-antibodies labeled with the different fluorophores.

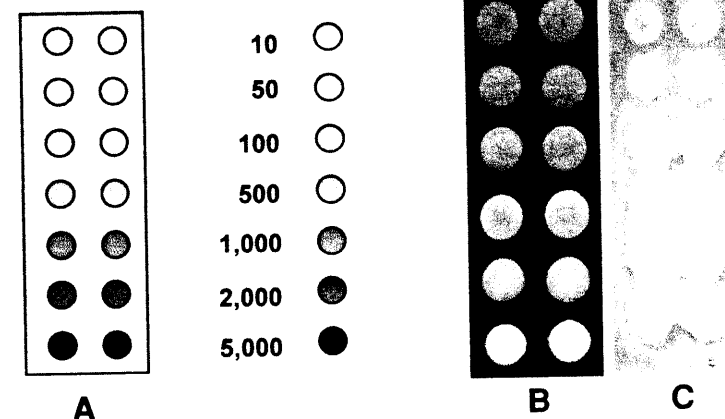


Fig. 3. Detection of IgE by the non-amplified and amplified protocols. (A) Schematic of IgE dilution curve. (B) Detection of IgE by Alexa 546-labelled anti-human IgE. (C) Detection of IgE by biotinylated anti-human IgE followed by HRP-streptavidin and Alexa 546 tyramide conjugate.

The slides are rinsed with wash buffer (five times 1 mL) and dried at 37°C prior to reading in the fluorescent scanner.

3.5. Processing of Microarrayed Slides for Determination of IgE in Human Serum

For this purpose, a protocol incorporating signal amplification has been identified and is described below (see **Subheading 3.5.2.**) and in **Fig. 3**. The amplification protocol is required to increase test sensitivity because human IgE circulates at very low concentrations.

3.5.1. Preliminary Procedures

3.5.1.1. SERUM SAMPLES

Undiluted serum samples are first analysed with a commercial ELISA (e.g., Radim S.p.A.). ELISAs are performed and samples categorized according to the manufacturer's instructions.

3.5.1.2. PREPARATION OF THE SLIDES FOR PROCESSING

See **Subheading 3.4.1.3.**

3.5.2. Processing of Slides

Slides are incubated with serum (150 μ L) for 60 min at room temperature and then thoroughly rinsed with wash buffer (five times in 1 mL). A 60-min incubation with a biotinylated anti-human IgE (150 μ L) at a final concentration of 1 mg/L in the appropriate diluent is performed at room temperature. After rinsing with wash buffer (five

times 1 mL), slides are incubated for a further 60 min at room temperature with a HRP-streptavidin conjugate diluted 1:100 in the appropriate diluent (*see Subheading 2.3.*). The resulting streptavidin-biotinylated antibody complexes are detected by incubating the slides for 15 min at room temperature with Alexa 546-tyramide conjugate, diluted 1:100 with Molecular Probes' diluent (namely amplification buffer) containing 0.015 mL/L hydrogen peroxide (*see Note 3*). On completion of the final processing step, the Gene-Frame is removed and slides are dried at 37°C for 10–15 min. Slides are then ready to be scanned.

Note: The HRP-streptavidin and Alexa 546-tyramide conjugates, hydrogen peroxide, and the amplification buffer are all provided in the TSA kit no. 23 supplied by Molecular Probes.

3.6. Scanning of Processed Slides

Scanning is performed using ScanArray™ software, according to the manufacturer's instructions. Images (**Figs. 1B** and **2B**) are optimized using fine-tuning capabilities provided (notably, adjustment of laser/photomultiplier tube [PMT] settings) and stored as bitmap files.

3.7. Quantitative Analysis of Image Data

Quantification is achieved using QuantArray™ software according to the manufacturer's instructions. Background signal and fluorophore crosstalk (if appropriate) are automatically subtracted.

Data are stored as Excel files. Calibration curves incorporated in each protein array are fitted using the appropriate curve-fitting routine and provide a basis on which to correlate the signal generated against a concentration of the analyte under determination. Signals from printed antigens are then interpolated from these calibration curves.

4. Notes

1. Printing solutions: Tween-20 is the surfactant of choice; print quality is much improved by the addition of 0.01% Tween-20 to all print solutions. PVP is used for printing some antigens because it retains a hydration shell surrounding the molecules in solution, allowing them to maintain their tridimensional structure. Sucrose is used for similar reasons; furthermore, sucrose is critical for the stability of the CMV antigen.
2. Signal generation: Fluorescent end points are used as the signal generation option of choice. Other end points have been described (such as colorimetric, chemiluminescent) but direct labeling of proteins with fluorophores to generate high specific activity and stable labels is easy to perform, and instrumentation is widely available for the determination of the emitted signal. It is preferable to choose fluorophores with spectral properties >500 nm because many proteins in blood fluoresce <500 nm, and background signals are much reduced at these wavelengths. The Alexa range of dyes (Molecular Probes) and the Cy dyes (Amersham Biosciences) can be recommended for this type of protein array immunoassay.
3. Signal amplification using tyramide reagentry: In the presence of hydrogen peroxide, HRP activates the dye-labeled tyramide derivatives creating extremely reactive short-lived tyramide radicals that bind to nucleophilic residues on the slide surface. This results in significant amplification of the bound signal. Use of this type of chemistry can increase signal generation by 50- to 100-fold.

Appendix

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