

Chapter 19

Microarrayed Allergen Molecules for Diagnostics of Allergy

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Summary

With the evolution of peptide synthesis techniques and microarray technology, it is now possible to map and characterize allergenic epitopes on a microarray platform. The peptide microarray-based immunoassay allows simultaneous analysis of thousands of target peptides using small volumes of diluted serum samples, and has a promising future to become a superior testing tool for aspects of food allergy diagnosis and prognosis, as well as for designing recombinant allergens for safe immunotherapy. Characterization of allergenic epitopes provides fundamental knowledge for understanding mechanisms of food allergy. This chapter describes in detail the development of a sensitive and reliable peptide microarray-based immunoassay. The information includes a comparison of different slide substrates, effect of buffers on spot morphology, performance of printing pins, immunolabeling detection systems with different levels of sensitivity, and suggested approaches to data analysis.

Key words: Microarray, Peptide array, Epitope mapping, Allergy diagnosis.

1. Introduction

Food allergy has emerged as an important public health problem in the United States (1). Approximately 4% of the American population and 6–8% of children <3-years old are affected by IgE-mediated food allergy, with symptoms ranging from mild oral pruritus to potentially life-threatening anaphylactic shock. Food allergy is currently diagnosed by clinical history and *in vivo* or *in vitro* confirmation of allergen-specific serum IgEs (2).

However, in many cases the history is uncertain and the efficacy of current testing is limited. First of all, positive IgE testing is not highly predictive of clinical allergy. Confirmation of clinical allergy, therefore, often requires oral provocation testing, which

carries some risk to the patient. This is not due to technical assay limitations, but follows from the fact that although allergen-specific IgE is necessary for these reactions, additional variables determine clinical reactivity and sensitivity. One of these variables is the quantity of specific IgE or the ratio of specific IgE to the total IgE pool. Numerous studies have documented the positive relationship between IgE quantity and the likelihood of clinical allergy (2), yet there is a large “grey zone” between high levels with good positive predictive values and undetectable levels that have good negative predictive values. In addition, the relationship between current IgE testing and the patients’ clinical sensitivity, usually defined as one of or a combination of reaction severity and the dose of allergen required to provoke a reaction, is very weak. Finally, another limitation of current testing is regarding the prognosis of a patient’s disease course. The majority of pediatric patients with some forms of food allergy (e.g., milk, egg, wheat, soy), will outgrow them during childhood. For at least some allergens there is again a positive but weak correlation between specific IgE level and duration of clinical allergy.

Recent studies have suggested that these aspects of clinical reactivity to food allergens may correlate better with features of allergen-specific IgE when looking at epitope-specific recognition. For example, patients with persistent allergy or with a history of more severe allergic reactions to milk (3, 4), peanut (5) and egg (6) were found to recognize a larger number of IgE epitopes. IgE epitope mapping may become an additional tool for allergy diagnosis and prediction. Additional research in larger prospective studies is underway to determine whether some of these correlations are stronger than those seen with current tests.

Characterization of allergenic epitopes is also of fundamental importance for understanding mechanisms of food allergy and for designing safe immunotherapeutics (7, 8). Previous efforts to treat food allergy using traditional allergen injection immunotherapy were suspended due to very high rates of iatrogenic anaphylaxis. Targeted alterations of the IgE epitopes is one strategy for reducing this risk while maintaining the proven efficacy of immunotherapy (9). Human trials using recombinant peanut allergens modified to alter IgE epitopes are beginning now and if successful, will likely usher in a wave of demand for trials of other food allergens.

In the past, spot membrane based immunoassay has been widely applied for epitope mapping (3, 10). In this system, the peptides are synthesized on the membrane and then incubated with the patient’s sera. However, synthesis of large numbers of peptides is relatively error prone, time consuming, labor intensive, and expensive. Immunoassay in this format also requires a relatively large volume of serum from each patient and is limited to the number of targeted peptides.

With the development of microarray technology (11–13) and evolution in peptide synthesis techniques (14, 15) we have been applying peptide microarray-based immunoassays for epitope mapping of several food allergens (16–19). There are several advantages of using microarray-based immunoassay for epitope mapping. It is possible to assay thousands of target peptides in parallel using small volumes of diluted serum samples, greatly reducing the biological sample cost of individual assays and allowing for more robust replication and statistical approaches to analysis and epitope determination. Several immunoglobulin subclasses other than IgE can be tested simultaneously, allowing us to simultaneously investigate potential regulatory responses (e.g., IgG4) that may influence clinical reactivity. In this chapter we will describe the methods for our preparation and processing of the peptide microarray-based immunoassay.

2. Materials

2.1. Microarray Printing Preparation

1. Glass slides coated with epoxide groups are from TeleChem International, Inc. (Sunnyvale, CA) (*see Note 1*). The slides should be used within 6 months of the manufacturing date and stored under vacuum before printing. Note the notch on the upper right corner of the slide indicating that samples should be printed on the side facing upward.
2. Peptides consisting of 15 or 20 amino acids length with an offset of three corresponding to the primary sequences of food allergens are commercially synthesized by JPT Peptide Technologies GmbH (Berlin, Germany). The synthesized peptides are either custom-made peptides supplied as lyophilized powder with purity over 70% (analyzed by HPLC and mass spectrometry) or peptides derivatives synthesized using the PepStar technique (*see Note 2*).
3. Protein printing buffer (PPB) (TeleChem International, Inc.).
4. Sodium lauryl sarcosinate (Sarkosyl) (Fisher Scientific, Fair Lawn, NJ).

2.2. Microarray Immunolabeling

1. Phosphate buffered saline (PBS) with Tween 20 (0.05%) (PBST): a 10× PBS solution is obtained from Roche Diagnostics Inc. (Indianapolis, IN) containing 0.01 M KH_2PO_4 , 0.01 M Na_2HPO_4 , 1.37 M NaCl, 0.027 M KCl, pH 7.0. Dilute 200 mL of 10×PBS with 1,800 mL of distilled water and add 1 mL of Tween 20.

2. Blocking buffer: 1% human serum albumin (HSA, Sigma, 85% purity) in PBST.
3. Secondary antibody for 1-step Alexa detection system: Polyclonal goat anti-human IgE (gift from Phadia, Uppsala, Sweden) and monoclonal mouse anti-human IgG4 (BD Pharmingen Inc., San Diego, CA) are conjugated with Alexa 546 and Alexa 647 (Invitrogen, Carlsbad, CA), respectively (*see Subheading 3.1*).
4. Secondary antibody for 2-step UltraAmp signal amplification system: Polyclonal biotinylated anti-human IgE (Kirkegaard & Perry Laboratories Inc., Gaithersburg, MD) and monoclonal anti-human IgG4-FITC (SouthernBiotech, Clone HP6025, Birmingham, AL).
5. UltraAmp detection reagents: Anti-biotin oyster 550 (350) and anti-FITC oyster 550 (350) and UltraAmp buffer are obtained from Genisphere Inc., Hatfield, PA. Salmon sperm DNA is from Invitrogen (Carlsbad, CA).
6. 15 mM Tris-HCl buffer, pH 8.0: 1 M Tris-HCl buffer, pH 8.0 is prepared by dissolving 121 g Tris base in 1 L distilled water and adjusting pH to 8.0 with concentrated HCl. Dilute 15 mL 1 M Tris-HCl buffer, pH 8.0 with 985 mL distilled water to make 15 mM Tris-HCl buffer, pH 8.0.

3. Methods

3.1. Preparation of Fluorescent Labeled Secondary Antibody

1. Concentration of secondary antibody: For the optimal conjugation with fluorochrome the concentration of antibody should be >2 mg/mL. Monoclonal antibodies are concentrated to >2 mg/mL using Amicon centricon concentrator YM30 (Amicon, Inc., Beverly, MA) according to manufacturer's instructions. The antibodies must be in a buffer free of primary amines, ammonium ions, sodium azide (>3 mM) and thimerosal (>1 mM), otherwise the conjugation reaction will be interfered. Buffer can be replaced (preferably by PBS) using dialysis.
2. Conjugation: A conjugation mixture is prepared as followed: 200 μ L antibodies/proteins (>2 mg/mL) + 20 μ L of 1 M NaHCO_3 + 20 μ L of 10 mg/mL Alexa546/647, and incubated at room temperature for 1 h with gentle stirring (protected from light). The fluorescent labeled antibody/protein is then loaded (120 μ L/column) onto the BioSpin 30 column (BioRad, Hercules, CA) and centrifuged at $1,000 \times g$ for 4 min. The eluate containing the purified fluorescent labeled

antibody/protein is collected and stored at 4°C protected from light. The conjugate should be stable in the presence of 2 mM sodium azide for several months.

3. Determination of degree of labeling: A 10 µL of the purified fluorescent conjugate is diluted 1/10, followed by serial dilutions 1/20, 1/40 and 1/80 with PBS and the absorbance of the diluted conjugates are measured in a 100 µL quartz cuvette with a 1 cm path length at both 280 nm (A_{280}) and either 650 nm (A_{650}) for Alexa 647 or 558 nm (A_{558}) for Alexa 546. The protein concentration in the diluted sample and the degree of labeling are calculated as follows:

For Alexa 546 conjugate:

$$\text{Protein concentration (M)} = \frac{(A_{280} - (A_{558} \times 0.12)) \times \text{dilution factor}}{203,000}$$

$$\text{Moles dye per mole protein} = \frac{A_{558} \times \text{dilution factor}}{104,000 \times \text{protein concentration (M)}}$$

For Alexa 647 conjugate:

$$\text{Protein concentration (M)} = \frac{(A_{280} - (A_{650} \times 0.031)) \times \text{dilution factor}}{203,000}$$

$$\text{Moles dye per mole protein} = \frac{A_{650} \times \text{dilution factor}}{239,000 \times \text{protein concentration (M)}}$$

The fluorescent conjugate usually has fluorochrome:antibody molar ratios of 5–10.

3.2. Printing of Peptide Microarrays

1. Preparation of printing plate: The lyophilized peptides are resuspended in dimethyl sulfoxide (DMSO) at 1 mg/mL, diluted 1/2 in 2× protein printing buffer (PPB) with 0.02% Sarkosyl (*see Note 3*) (**Fig. 1**), and transferred (10–15 µL/well) into the printing plate (assay plate 384 well, low volume, nonbinding surface round bottom, white polystyrene, Corning). The printing plate is covered with Robolid (Corning), centrifuged at 300 × *g* for 5 min, and stored in a sealed plastic bag at 80°C until use. The centrifuge step is to remove any possible bubbles in the wells and it is very important.
2. Microarray printing instrument: NanoPrint Microarrayer 60 (TeleChem International, Inc.), a contact microarray printer, is used for peptide printing. It is equipped with 2 × 4 ArrayIt Stealth Micro Spotting Pin (SMP3B), which allows pick up of sample solutions (~0.6 µL) from the printing plate and deposition of a specific amount (~0.9 nL) by touching the slide surface. The printing consistency of these eight pins have

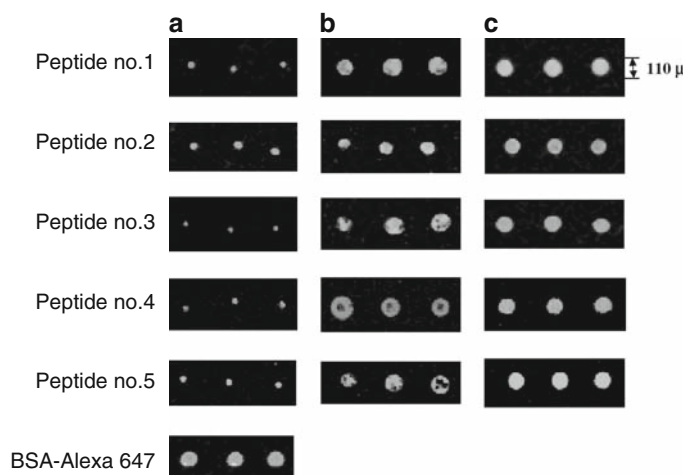


Fig. 1. Effects of buffers on spot morphology. The triplicate spots of five milk peptide samples on the slides were immunolabeled with a 1/5 diluted serum pool (specific IgE to milk: 850 kU_A/L) of five milk allergic patients using the Alexa detection system as described in **Subheading 3.3**. The images taken were scanned with red laser (for Alexa 647). The triplicate spots of BSA-Alexa 647 (1.5 μg/mL in PPB) are also shown as a comparison. (a) peptides printed as 0.33 mg/mL in PPB, 33% DMSO, (b) peptides printed as 0.33 mg/mL in PPB, 33% DMSO, 0.02% Sarkosyl (c) peptides printed as 0.5 mg/mL in PPB, 50% DMSO, 0.02% Sarkosyl.

been tested in the experiment in which the eight different pins showed the ability to deposit the same amount of samples and print at least 250 consistent spots of a fluorescent labeled protein without sample reloading (*see Fig. 2*). Therefore the pins are usually used to print not more than 200 spots from each loading. With each loading, the first two printed spots are usually very large and will not be used (set of two dummy slides). If possible, it is better to keep the microarray printing facility in a clean room to avoid any dirt or dust that gets into the printing plate or onto the slide surface during printing.

3. Microarray printing: The whole printing process is controlled by the NanoPrint™ Microarray Manager Software (TeleChem International, Inc.). Peptide samples are usually printed in triplicate and approximately 1/4 of the printed spots are PPB alone (>400 spots/array) which are used as negative control for background normalization. Alexa 546 and Alexa 647-labeled bovine serum albumin elements (prepared following antibody conjugation protocol, *see Subheading 3.1*) are used for the purpose of grid alignment. Other proteins, such as different concentrations of purified human IgE, may be printed as well as positive controls. The humidity is maintained at 55–65%

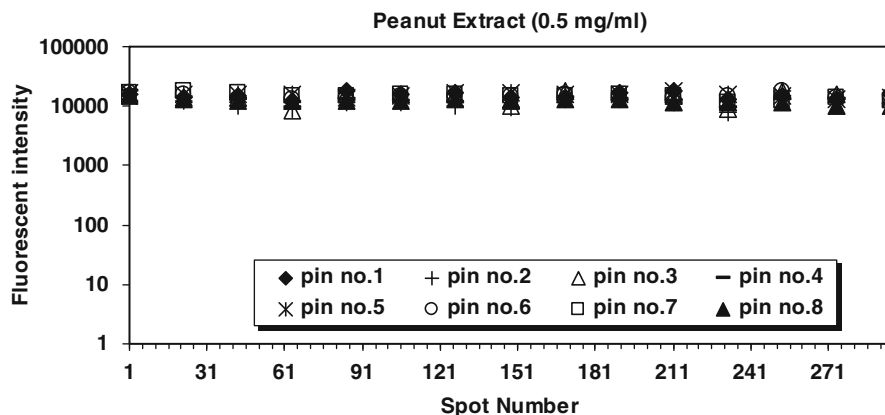


Fig. 2. Pin performance test. 288 spots of 0.5 mg/mL peanut extract were printed from each of the eight spotting pins in a row without reloading. One printed slide was then immunolabeled with the serum of a patient with high specific IgE (>100 kU_A/L) to peanut using the Alexa detection system (*see Subheading 3.3*) and scanned with the green laser (*see Subheading 3.4*). The fluorescent intensity (dFU) shown here is the median fluorescent intensity of the spot subtracted by local background.

during printing to avoid sample evaporation (*see Note 4*). All array elements are printed in duplicate (two sets of triplicates) to improve precision, and to determine intra-assay variation. After the first set of triplicate spots are printed, the plate is turned around so that the second set of triplicate spots are printed using different pins to minimize the possible effect of printing inconsistency of different pins. In order to save slide space and serum volume used for immunolabeling, two arrays are printed on a single slide. Printed slides were dried overnight in the printer (at low humidity) to allow full binding to the slide substrate, stored at room temperature (*see Note 5*) and used within 2 months. A .gal-file containing information on sample location and identification from each printing is generated using the Microarray Manager Software.

After printing, it is recommended to perform quality control (QC) scan on dried slides using lower resolution for faster process (*see Note 6*). The purpose of QC scan is to monitor the quality and reproducibility of the printing as well as to serve as a reference before labeling.

3.3. Immunolabeling with Patient Serum

The printed arrays are incubated with sera. Sera of at least ten negative controls, either nonatopic people or atopic patients but not allergic to the same food should be run in parallel. Depending on the application, either 1-step Alexa detection system or 2-step UltraAmp could be applied (*see Note 7*) (**Fig. 3**).

1. Slide preparation: A rectangular incubation area around the printed arrays is demarcated using a hydrophobic Dako

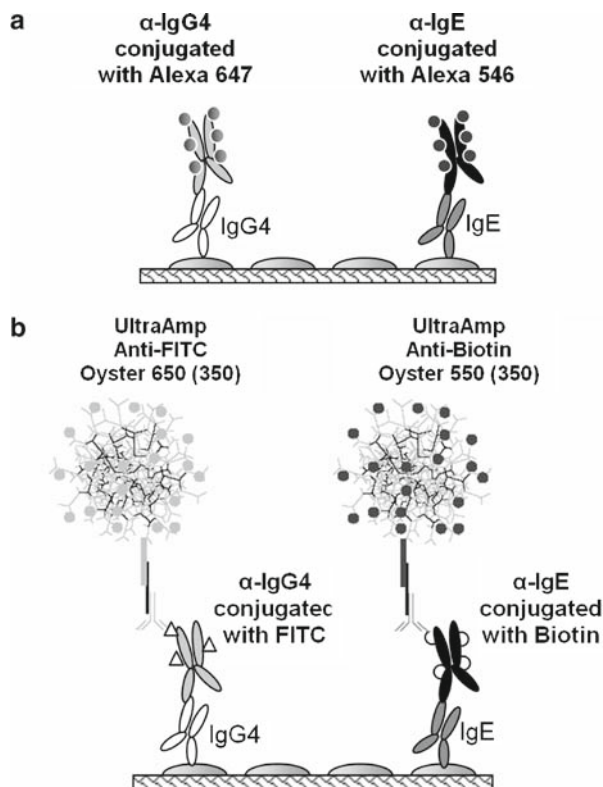


Fig. 3. Immunolabeling procedure. (a) 1-step Alexa detection system (b) 2-step UltraAmp amplification system.

Cytomation Pen (Glostrup, Denmark) which separates two arrays on the same slide. This step must be done at least 3 h before immunolabeling (better overnight) to ensure that the ink is totally dry. All the incubation steps are performed in a humidity chamber (Binding Site, Birmingham, UK) covered by a black cloth on a rotating platform (Lab-Line Instruments Inc., IL) with gentle agitation. It is very important to keep the slide covered with solution at all times, never let it dry during the whole immunolabeling procedure.

2. Blocking and serum incubation: The slides are placed into the humidity chamber, secured by a magnetic strip, and rinsed with PBST. After removing PBST from the slide surface by aspiration at the array corner, 400 μ L of blocking buffer are added onto each array to block the nonspecific binding sites, and incubated for 1 h at room temperature. The volume used for incubation depends on the size of the array area. Usually 200–400 μ L is enough to cover one array (half a slide). If the volume cannot spread out and cover the slide surface by itself,

one can try to spread it out by pipette tips held longitudinally but never touched the slide surface. Patients' sera are prepared by diluting 50 μ L of serum with 200 μ L of blocking buffer (*see Note 8*) and applied onto the slide after the blocking buffer is removed by aspiration. As the two arrays are separated by the hydrophobic pen, two different sera can be applied on the same slide. The slides are incubated with the sera for overnight at 4°C (*see Note 9*) and washed carefully and thoroughly (5×1 min) with PBST. Avoid contamination between different arrays especially arrays on the same slide.

3.3.1. Detection of IgE Binding Using 1-Step Alexa Detection System

The slides are incubated (250 μ L/array) for 1 h at room temperature with a cocktail of Alexa 546 conjugated polyclonal goat anti-human IgE and Alexa 647 conjugated monoclonal mouse anti-human IgG4, both of which diluted 1/5,000 (*see Note 10*) in blocking buffer. The slides are washed with PBST (3×1 min), distilled water (3×1 min), and spin dried using a bench top slide spinner (TeleChem International, Inc.).

3.3.2. Detection of IgE Binding Using 2-Step Ultraamp Amplification Detection System

The slides are incubated (250 μ L/array) for 1 h at room temperature with a cocktail of polyclonal biotinylated anti-human IgE (*see Note 11*) and monoclonal anti-human IgG4-FITC, both diluted 1/500 in the blocking buffer (*see Note 11*). Slides are washed with PBST (3×1 min), incubated for 4 min with 1 mM ethylene diamine tetraacetic acid (EDTA) in PBST, washed again with PBST, equilibrated for 1 min with UltraAmp buffer and, after aspiration, incubated 3 h at room temperature with a cocktail of anti-Biotin Oyster 550 (350) and anti-FITC oyster 550 (350) in UltraAmp buffer at concentration of 0.6 μ g/mL, each with addition of 0.02 μ g/mL of salmon sperm DNA. The slides are washed with PBST (3×1 min), rinsed with 15 mM Tris-HCl buffer, pH 8.0 (30 s, this step helps to retain signal intensity), spin dried, followed by wash with 0.05 $\times$ PBS (30 s), and finally spin dried.

3.4. Microarray Scanning and Slide Alignment

Reactions of serum IgE to each peptide sample are represented by the fluorescent intensity of the peptide spot.

1. Scanning: The immunolabeled slides are scanned with both green (laser excitation wavelength at 543 nm for Alexa 546) and red lasers (laser excitation wavelength at 633 nm for Alexa 647) using a ScanArray®Gx (PerkinElmer, Waltham, MA) equipped with the program ScanArray Express (Perkin Elmer). Adjust the laser power and PMT gain as high as possible without saturating the signal of any spot (*see Note 12*). For image resolution use a resolution of no more than 20 μ m (*see Note 6*). Keep the same setting for the slides from the same project. Scanned images at both wavelengths are saved as TIF files.

2. Alignment: The .gal-file is loaded and the scanned images are quantitated with ScanArray Express using the “adaptive threshold” (*see* **Note 13**) quantitation algorithm to generate a spread sheet containing values for median/mean signal intensity, median/mean background intensity, background intensity standard deviation, and signal-to-noise ratio for each spot on the array. The boundary of each spot are carefully checked and re-adjusted if it is not located on the real spot due to possible artefacts. The spread sheets are exported and saved as comma-delimited text files.
3. Data analysis: Data are analyzed with R programming language (<http://www.r-project.org/>) version 2.6.0 as followed: Briefly, the read out (S) used for each spot, including the replicates for peptides and PPB, is the median fluorescent signal of the spot divided by local background and $\log_2$ transformed. A Z -score is calculated for each array element (spot) using PPB values within the same array:

$$Z_i = \frac{S_i - \text{Median}(S_{\text{PPB}})}{\text{MAD}(S_{\text{PPB}})}$$

4. Where S_i is the read out for the array element transformed into Z score (Z_i); and the $\text{median}(S_{\text{PPB}})$ and $\text{MAD}(S_{\text{PPB}})$ are the median and the median absolute deviation (MAD) of all the read outs of PPB spots, respectively.

The total Z value for each peptide is the Median of Z -scores of the six replicate spots. As the peptides are overlapped by 12 or 17 amino acids, which means overlapping peptides might share the same epitopes, a weighted average of Z value could be calculated by the formula $Z = 0.25 * Z_{-1} + 0.5 * Z_0 + 0.25 * Z_{+1}$ and subsequent analysis is carried out based on the weighted average index. An individual peptide sample is considered positive if its index exceeds 3. For comparison of Z -scores between groups, a Wilcoxon test is applied ($p < 0.01$).

4. Notes

1. Printing slides with various substrates, with either 2- or 3-dimensional surfaces, are commercially available now (20). We have tested slides with aldehyde substrates and found that they have less binding capacity. We have also tested the 3-dimensional nitrocellulose substrate slides such as FAST slide from Whatman and SuperNitro slide from TeleChem International, Inc. They are more difficult to handle and

usually show high background. Moreover, the presence of organic solvents, even as low as 20% DMSO, which is necessary to maintain peptide solubility, damages the nitrocellulose membrane.

2. Depending on the specific project, it might be possible to use the peptide synthesized using the PepStar technique from JPT Peptide Technologies GmbH (Berlin, Germany). The synthesized peptide derivatives carry a reactivity tag and a linker at their N-terminus allowing chemoselective immobilization. It provides much less amount of peptides (50 nmol for each peptide) but at much lower cost for each peptide and faster production time.
3. It is desirable to keep peptides in high concentration of organic solvents, such as dimethylsulfoxide (DMSO) and acetonitrile, which may increase peptide solubility and prevent peptide aggregation. In addition, some synthetic peptides seem to form very small spots after printing. We have solved this problem by adding 0.02% Sarkosyl and increasing DMSO concentration from 33 to 50% (**Fig. 1**).
4. We have found that the signal intensities of the spots of the same fluorescent samples are higher when printed at higher humidity, possibly by increasing the reactivity of the epoxide substrate on the slide or more materials getting deposited. Therefore it is desirable to keep the humidity stable but as high as possible during printing.
5. Although it has been suggested by some researchers to store the printed slides *in vacuum* and store the arrays in refrigerator, we found that storing the printed slides at room temperature may minimize the nonspecific binding (noise) to the slide background, possibly by deactivation of the remaining active epoxide substrate on the slide surface.
6. A quick scan can be performed using lower resolution (high pixel size such as 50 μm) to check the printing quality or locate the area with the printed spots before a second scan with higher resolution is performed.
7. Both of these two detection systems have their own advantages and disadvantages: For the 1-step Alexa detection system, the Alexa fluorochrome and secondary antibody usually form stable conjugate with fluorochrome:antibody molar ratios of 5–10. Our results have shown that it gives very reproducible results ($R > 0.90$) regardless of printing lots and immunolabeling day but the sensitivity is not high enough for allergic patients with less than 5 $\text{kU}_\text{A}/\text{L}$ of specific IgE level. The UltraAmp amplification detection system, on the contrary, contains 5–15 specific antibody molecules and 350 Fluorochromes on each UltraAmp molecule.

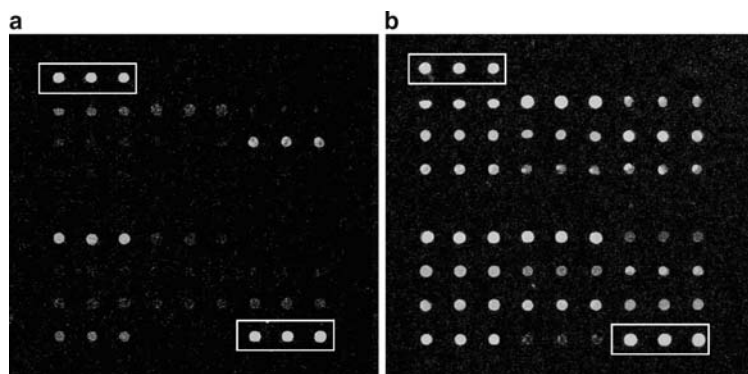


Fig. 4. Comparison between 1-step Alexa detection system and 2-step UltraAmp amplification detection system. In this experiment, replicate milk peptide arrays were immunolabeled with a 1/200 diluted serum pool (specific IgE to milk: 850 kU_A/L) of five milk allergic patients using either the Alexa detection system (a) or the UltraAmp detection system (b) as described in **Subheading 3.3**. The images shown here are the scanned images (green laser for Alexa 546) of one subarray of each array. The peptides were printed as triplicate spots and the spots of positioning control BSA-Alexa 546 (1.5 µg/mL in PPB) are indicated in the white frames.

Therefore it can greatly (*see Fig. 4*) increase the fluorescent signal intensity of the bound secondary antibody and be applied for allergic patients with specific IgE level even less than 0.35 kU_A/L. However, the reproducibility so far we can achieve ($R = \sim 0.75$) is lower than Alexa detection system due to high sensitivity and high susceptibility to variations in handling. In addition, the UltraAmp reagents cost much more than the Alexa reagents.

8. It is very important to vortex the serum before mixing with the blocking buffer. Keep the serum and the diluted serum on ice during preparation.
9. In order to decide the optimal incubation time, we have tested several serum incubation conditions (1 h at 37°C, 4 h at room temperature, and overnight at 4°C) using replicate arrays and found that overnight at 4°C allows more positive binding while not creating too much nonspecific binding and background noise.
10. The working concentration of the secondary antibody is in the range of 0.4 µg/mL for polyclonal antibody to 2 µg/mL for monoclonal antibody. However, a serial dilution of the secondary antibody should be tested in preliminary experiment to decide the optimal dilution factor which has the best balance between sensitivity and specificity.
11. As an option, monoclonal biotinylated anti-human IgE could be used to increase specificity. In addition, a cocktail of

several clones can be applied to compensate steric hindrance effect.

12. Higher laser power is usually desirable for scanning. If the pixels have been saturated (beyond the detection limit of the scanner) it will appear as white-color pixels.
13. There are three other quantitation methods: adaptive circle, fixed circle, and histogram. The main advantage of the adaptive threshold method is that it can adapt to different spot morphology better than the other methods. Please read "ScanArray Express Microarray Analysis System User Manual" for more details of these four methods.

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