

IgE Epitope Mapping Using Peptide Microarray Immunoassay

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Abstract

IgE epitope mapping of food allergens contributes to a better understanding of the pathogenesis of food allergy and may become an additional tool for food allergy diagnosis/prognosis. Microarray platforms which allow for simultaneous screening of a large number of peptides corresponding to the sequences of food allergens are ideally suited for large-scale IgE epitope mapping. Here we describe the method of performing a reliable and sensitive peptide microarray immunoassay, which was developed in our lab and results in the identification of candidate IgE epitope biomarkers useful in determining allergic disease severity and prognosis, as well as in the prediction of treatment outcomes. A gastric digestion model that can be applied to prescreen peptides and reduce costs in the peptide microarray is also described in this chapter.

Key words IgE, Food allergy, Epitope mapping, Peptide microarray, Gastric digestion, Immunoassay

1 Introduction

Food allergy is usually mediated by immunoglobulin E (IgE) antibodies through their binding to specific IgE epitopes within allergenic proteins. Identification of IgE-binding epitopes in food allergens will contribute to the design of well-tolerated immunotherapeutic agents and a better understanding of the pathogenesis of food allergy. In addition, studies have suggested a role for sequential IgE-binding epitopes as biomarkers for characterizing various phenotypes of food allergy [1].

Due to its ability to evaluate thousands of targets in parallel by using small volume of sample, the peptide microarray immunoassay is ideally suited for large-scale epitope mapping of IgE antibodies [2]. A typical peptide microarray consists of two experimental steps: printing a library of overlapping peptides, corresponding to the amino acid sequence of the target protein(s) onto glass slides coated with a functional layer (i.e., epoxy, aldehyde) where the peptides bind covalently to the functional groups on the slide surface; and

immunolabeling of the printed slides with patients' sera. The use of overlapping peptides printed in the microarray format increases the mapping resolution and the accuracy of the test. In the past 10 years, we have developed and optimized a reliable and sensitive peptide microarray immunoassay. Our optimized peptide microarray platform is capable of generating reproducible data with high correlation coefficients ($r > 0.9$) [3], and detecting IgE binding from diluted serum sample with specific IgE levels as low as 0.068 kUA/L [4]. Using our peptide microarray, we have successfully mapped the sequential IgE-binding epitopes for a variety of food allergens, such as allergens from peanut, milk, lentil, and shrimp [5–8], and identified several IgE epitopes as candidate biomarkers for allergy diagnosis/prognosis [4] or tolerance prediction [9].

Synthesizing and printing a large library with hundreds/thousands of overlapping peptides covering entire protein sequences involves a high cost and also limits the number of food allergens that can be tested in one microarray. A selected peptide library covering only a portion of protein sequences with high probability of being epitopes can be an alternative to reduce the expenses and increase the number of allergens/foods that can be tested. In order to induce sensitization and elicit an allergic response, allergenic proteins must retain sufficient structural integrity after digestion [10, 11]. The sequences resistant to gastric digestion have higher chances of being bound by IgE antibodies. Therefore, a simulated protein digestion method mimicking physiological conditions of the stomach during gastric digestion [12] is useful as a prescreening of allergen sequences to select digestion-resistant sequence for peptide microarray.

In this chapter, we describe the method of IgE epitope mapping using peptide microarray immunoassay. This method is not only useful for epitope mapping of IgE antibodies, but can also be applied to other immunoglobulin subclasses (i.e., IgG4) by switching to or adding (for concurrent testing) the corresponding secondary antibody. In addition, as an option to downsize the peptide library for each allergen, we provide the method of performing simulated protein digestion with detailed description of the preparation, processing, and hydrolysis of allergenic proteins.

2 Materials

2.1 Gastric Digestion

1. Phosphatidylcholine (PC) (Larodan, Malmo, Sweden).
2. Simulated gastric fluid (SGF), containing 35 mM NaCl, pH 2.0: SGF is prepared by dissolving 1 g of NaCl in 0.5 L of distilled water (dH₂O) and adjusting pH to 2.0 with 1 N HCl.
3. Porcine pepsin (Sigma-Aldrich, St. Louis, MO, USA): EC 3.4.23.1, 3640 U/mg protein.
4. RP-HPLC-MS/MS access is required for final analysis of gastric digestion.

2.2 Peptide Microarray

1. SuperEpoxy 2 (SME2) glass slides (Arrayit Corporation, Sunnyvale, CA, USA), used for all peptide microarray printings (*see Note 1*).
2. A library of overlapping peptides (*see Note 2*) (JPT Peptide Technologies GmbH, Berlin, Germany), consisting of 15 amino acids (AA) overlapping by 12 (3-offset), corresponding to the primary sequence of the target proteins, are commercially synthesized either as custom-made or PepStar format (*see Note 3*). If enzymatic digestion has been performed for protein sequence pre-screening, only peptides covering the digestion-resistant sequences are commercially synthesized for printing.
3. 384-Well, round-bottom, non-sterile assay plates (Corning Incorporated, Corning, NY, USA) and lids are required for proper storage and printing of the peptides (*see Note 4*).
4. NanoPrint 60, equipped with 2×4 Stealth Micro Spotting Pins (SMP3B) and controlled by the NanoPrint Microarray Manager Software (Arrayit Corporation), is used to print peptide libraries (*see Note 5*).
5. Hybridization Chamber/Stain Tray (Worldwide Medical Products, Bristol, PA, USA), used for all the incubation steps in the immunolabeling procedure. We recommend purchasing the tray with the black lid, as some of the incubation steps in this assay require protection from light sources.
6. Dako Pappen (Dako North America Incorporated, Carpinteria, CA, USA).
7. High-throughput wash stations and squeeze bottles (Arrayit Corporation).
8. Dimethyl sulfoxide (DMSO), 99.7+%, extra dry over molecular sieve (Acros, New Jersey, USA).
9. Phosphate-buffered saline (PBS) stock solution: For peptide microarray washing steps, 1×, 0.1×, and 0.05× solutions are prepared by diluting the stock solution in distilled water 1:10, 1:100, and 1:200, respectively.
10. Phosphate-buffered saline-Tween (PBS-T): 1 mL Tween 20 is diluted with 1× PBS to a final volume of 2 L.
11. Ethylenediaminetetraacetic acid (EDTA), 1 mM in PBS-T: First, a 0.5 M stock solution of EDTA is prepared by dissolving 186.12 g in 800 mL dH₂O, adjusting pH to 8.0 by adding approximately 20 g sodium hydroxide (NaOH) while stirring, and adjusting the volume to 1 L with additional dH₂O. Second, the 1 mM solution is prepared by diluting 100 μL of the EDTA with PBS-T to a final volume of 50 mL.

12. 15 mM Trizma hydrochloride (Tris-HCl) buffer solution, pH 8.0: 30 mL of 1 M Tris-HCl, pH 8.0, is diluted with dH₂O to a final volume of 2 L.
13. Protein printing buffer (PPB), 2× solution (Arrayit Corporation), is used in the working plates to dilute the peptides. This reagent is essential for quality printings and provides efficient coupling and consistent spot morphology.
14. Blocking buffer: 1 % human serum albumin (HSA) in PBS-T is used to block all unbound sites on the slide surface prior to incubation with patient serum samples. It is also the diluent for both serum and detection antibodies.
15. IgE immunolabeling solution: 250 µL of solution containing monoclonal biotin mouse anti-human IgE (BD Biosciences, San Jose, CA, USA) and monoclonal biotin mouse anti-human IgE (ε-chain specific) (Molecular Probes by Life Technologies, Grand Island, NY, USA), each diluted 1:250 in blocking buffer.
16. Dendrimer signal amplification system: 200 µL of solution containing 12 µL UltraAmp Anti-Biotin oyster 550 (Genisphere Incorporated, Hatfield, PA, USA), 4 µL salmon sperm DNA (10 mg/mL) (Agilent Technologies, Santa Clara, CA, USA), and 184 µL UltraAmp Binding Buffer II (Genisphere Incorporated).
17. ScanArray Gx Microarray Scanner (PerkinElmer, Waltham, MA, USA).

3 Methods

3.1 Gastric Digestion

1. Phospholipid vesicle solution is prepared by dissolving PC in SGF at a concentration of 9.58 mg/mL. The mixture is sonicated until clear to the eye (~5 min). This reduces the crude lecithin solution into a suspension of single-shelled liposomes.
2. Solutions are then filtered through a 0.22 µm filter to remove any possible contaminants.
3. 39 mg of protein is dissolved in 3 mL of SGF (final concentration of 13 mg/mL). The pH is adjusted to 2.0 with 1 N HCl. Additional SGF is then added to bring the volume to 3.1 mL.
4. Phospholipid vesicle solution (3.6 mL) is then added to the protein mixture (9.58 mg PC/mL SGF) and incubated for 5 min at 37 °C.
5. Following the incubation, 6.030 mL of mixture is transferred to a clean tube and 303 µL of pepsin solution (18,200 U/mL SGF) is added to obtain the physiological ratio of 182 U/mg of protein.

6. Gastric digestion is performed in a shaking incubator for 60 min at 37 °C. The reaction is stopped by increasing pH to 7.5 with NaHCO₃. Distilled water is added to the sample until a final volume of 7.800 mL to obtain a final concentration of protein of 4.5 mg/mL. RP-HPLC-MS/MS analysis of the digested samples is performed in order to identify the sequences resistant to digestion.

3.2 Printing of Peptides

1. Working plates containing peptides in printing buffer (3 µL peptide + 3 µL 2× PPB) are stored at −80 °C, and left at room temperature for 10–15 min prior to use. Contents of the working plates are mixed well by placing the plate on a pedestal for 60 s in an ultrasonic bath, and then centrifuged at 130×*g* for 3 min at room temperature.
2. Peptides are printed from the working plate onto the SME2 slides in two sets of triplicate spots. Each slide can fit two or three full microarrays, depending on the size of the peptide library or the nature of the experiment (Fig. 1a–c). In addition to the peptides, 20 % of the printed spots must be “blanks” composed of 50 % v/v DMSO in PPB.
3. All printings are carried out under 55–65 % humidity in order to prevent any evaporation from the samples on the working plate. Once completed, humidity is turned off and slides are left to dry overnight in the printer to ensure that peptides are bound to the slides.
4. Following the drying period, the printed slides are scanned for quality check (QC) at low resolution (20 µm) to ensure proper peptide spotting. The microarrays on the printed slides are then outlined with the Dako pap pen and labeled with the printing and slide number (Fig. 1d). Marking the outline of all the arrays in the same sized square shape with a hydrophobic pen ensures proper hybridization of antibodies and amplification molecules to peptides, and also prevents cross-contamination between patient samples on the arrays.
5. All slides are then washed twice, each for 20 min in PBS-T and 0.05× PBS in high-throughput wash stations. This washing step removes excess, unbound peptides, leaving only the covalently bound peptides that are not visible by eye (Fig. 1e). In addition, this pre-wash reduces nonspecific binding and background noise. Slides are then dried in a centrifuge using the same settings as in step “A”—130×*g* for 3 min at room temperature. The immunolabeling protocol can now be initiated, or the slides can be stored in vacuum for up to 3 weeks.

3.3 Immunolabeling Protocol

1. Blocking: Slides are placed in the hybridization chamber, 250 µL of blocking buffer is added to each array (*see Note 6*), and gentle rotation for 1 h at 31 °C is required. The blocking buffer is then carefully removed by aspiration.

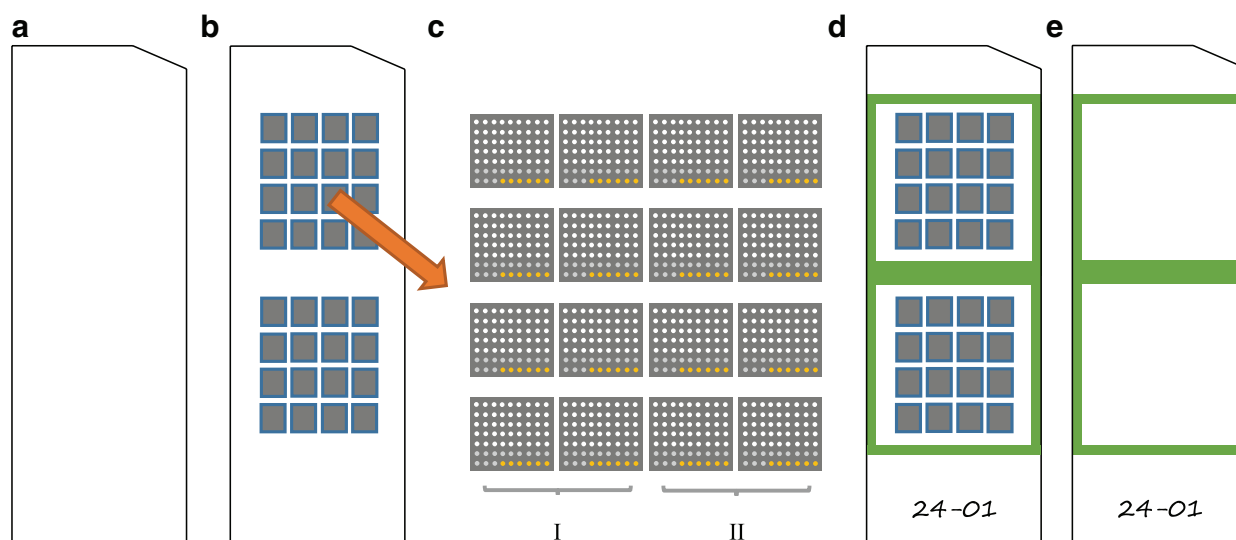


Fig. 1 (a) Blank SME-2 slide from Arrayit Corporation, the notch on the *top right* is used as a guide for holding the slide *right side up*. (b) Slide with two identical arrays after printing. Depending on the size of the peptide library, changes can be made to fit up to 3 arrays (for 3 individual serum samples) per slide. (c) Close-up view of 1 array. The *white* spots represent individual peptides, printed in triplicates. The *light gray* spots indicate “blank” PPB spots used for background normalization. It is important to note that at least 20 % of the array must be composed of PPB spots. The *yellow* spots, usually printed at the *bottom right* corner of each sub-array, represent the fluorescent-labeled BSA spots used as a grid control during alignment. Sub-panel I and sub-panel II are duplicates. Since each peptide is printed in triplicate, the total number of spots per peptide is 6. (d) After QC scan, printed slides are marked with a pap pen, enclosing the separate arrays in equal-sized squares for optimal hybridization. The center line must be thick enough to prevent cross-contamination between samples. Each slide is also labeled with the printing and slide number. (e) After washing the slides with PBS-T and 0.05× PBS, the arrays will no longer be visible by eye

2. Serum incubation: Patient serum samples (*see Note 7*), diluted 1:5 in blocking buffer, are added to the arrays (250 μL /array) and gently rotated overnight at 4 °C. The hybridization chamber must be always protected from light. Following overnight incubation, serum is removed by gently tapping the slides horizontally—this helps prevent cross-contamination between the vertical arrays. The slides are transferred onto a slide rack, dipped two times in PBS-T, and placed in a high-throughput wash station containing fresh PBS-T for 10 min at room temperature. The slides are then placed back into the hybridization chamber and the remaining PBS-T is removed by aspiration.
3. Secondary antibody incubation: For IgE detection, a fresh preparation of the biotinylated secondary antibody cocktail in blocking buffer is required for every experiment. The BD and Life Technologies antibodies are each diluted 1:250. After removing PBS-T from the slides by aspiration, 250 μL of the antibody cocktail is added onto each array, spread across the array outline, and the slides are gently rotated at 31 °C for 4 h

(*see Note 8*). The slides are quickly washed with PBS-T from a squeeze bottle, and after clearing the arrays with aspiration, 300 μL of 1 mM EDTA (*see Note 9*) in PBS-T is added to each array. This is then removed after rotating for 4 min at room temperature, followed by another quick PBS-T wash. The remaining PBS-T is removed by aspiration.

4. Dendrimer amplification: The next step in the immunolabeling protocol is the addition of dendrimers for signal amplification to increase the sensitivity of the assay. 200 μL of dendrimer buffer is added, spread across the arrays, and is left to sit on the slides for 1 min before it is removed. The dendrimer cocktail (200 μL /array: 12 μL anti-biotin dendrimer, 4 μL salmon sperm DNA, and 184 μL dendrimer buffer) (*see Note 10*) are added onto each array, followed by gentle rotation for 3 h at 31 °C, protected from light.
5. Final wash: The final wash aims to remove unbound dendrimer molecules and reduce excess salt deposits on the array surface that may interfere with the subsequent analysis. With the slides still in the hybridization chamber, they are washed quickly with PBS from a squeeze bottle, then dipped ten times in 15 mM Tris-HCl. Slides are dried in a centrifuge, and then dipped five times in 0.1 $\times$ PBS. After another spin in the centrifuge, the slides are dipped five times in 0.05 $\times$ PBS, and dried again before they are scanned for analysis. Dried slides may also be stored in vacuum for up to 5 days before scanning without compromising signal intensity.

3.4 Slide Scanning

The processed slides are scanned using a ScanArray®Gx (PerkinElmer, Waltham, MA, USA) microarray scanner. The difference between this scan and the original QC scan after printing is that the scanner settings are changed to a much higher resolution (10 μm) in order to increase sensitivity and detect even low-intensity IgE binding. Scanned images are saved as TIF files, and the fluorescence signals are digitized with ScanArray Express software (Perkin Elmer) using an alignment procedure (*see Note 11*). The aligned data for the arrays are then exported as comma-delimited text (.csv) files.

3.5 Data Analysis

- (a) A readout (S) for each array element (spot), including the replicates for peptides, controls and blanks (PPB + DMSO), is calculated (as indicated in the equation) by $\log_2$ transformation of the ratio of the median fluorescent signal of the spot to the median fluorescent signal of the local background:

$$S_i = \text{Log}_2 \left(\frac{\text{Median}(\text{Spot})}{\text{Median}(\text{Local background})} \right)$$

- (b) The S_i for each array element is transformed into a Z score (Z_i) by using the equation below:

$$Z_i = \text{Log}_2 \frac{S_i - \text{Median}(S_{\text{Blank}})}{\text{MAD}(S_{\text{Blank}})}$$

The $\text{Median}(S_{\text{Blank}})$ and $\text{MAD}(S_{\text{Blank}})$ are the Median and the Median Absolute Deviation (MAD) of all the readouts of the blanks spots, respectively. MAD is a robust version of the standard deviation (*see* **Note 12**). An index Z value of each peptide element is generated from the Median of Z scores of the six replicate spots. An individual peptide sample is considered positive if its index Z exceeds 3, meaning that the signal is above the background noise plus three times the MAD. As MAD is a robust version of the standard deviation, this means that the signal is significantly above the background with p value less than 0.003.

We perform the above transformation using R programming language (<http://www.r-project.org/>), but it can also be calculated using other software which does not require programming, such as Microsoft Excel. Further data analysis and presentation can be performed using R programming or other commercially available software such as Microsoft Excel, GraphPad Prism, Sigmaplot and TIGR Multiexperiment Viewer (TMeV).

4 Notes

1. SME2 slides by Arrayit Corporation provide high-quality surface chemistry necessary for proper peptide printing, binding, and subsequent immunolabeling. We choose this particular product due to their consistency in performance, 6-month shelf life, and minimal slide-to-slide variation. Once the slides are received, they must be stored in a cool, dry place at room temperature, and used within 6 months of the manufacture date.
2. Using overlapping peptides facilitates both the mapping of epitope sequences and the identification of key amino acids based on IgE binding intensity. In addition, peptides can form secondary structures and epitope sequences of solid-phase bound peptides may not always be exposed and available for binding with IgE antibodies. By using overlapping sequences, we increase the chances of detecting all epitopes.
3. Custom-made peptides are synthesized and aliquoted as lyophilized powders. The purity of these peptides has been measured by HPLC. In general, >70 % purity is required for peptide microarray printing. These peptides are resuspended in DMSO at 2 mg/mL concentration. The PepStar format peptides dif-

fer from custom-made in that the former is immobilized on the glass slides via a flexible linker at the N-terminus, and is provided already in solution in 96- or 384-well stock plates. All peptides (stocks and working plates) must be stored at -80°C until used.

4. Plates from Corning Incorporated are used as working plates. They have the proper dimensions for accurate printing, and are used to store peptides diluted in printing buffer.
5. Specific printing conditions are required in order to ensure performance and accuracy in the immunoassay. Having a clean workspace, preferably an isolated room to avoid dust buildup and other printing irregularities is very important. Humidity is also an important factor. The humidity level inside the printing chamber should be maintained around 60 % during printing to prevent the evaporation of peptides, and humidity in the room must not exceed 50 %, as this contributes to “pin sticking” and other technical problems with the printing unit. In addition to these conditions, the printer is equipped with a sonicating bath station for washing of the pins before and after printing, as well as in between visits to the working plate. The sonication station is filled with a 10 % ethanol solution, and is used for pin washing for 5 min before and after printing, and for 2 min for in-between visits to the plate. These washes are necessary for optimal cleaning and maintenance of the pins, prevent cross-contamination of peptides, and remove any particles that may interfere with accurate spotting.
6. For this step, and for all subsequent steps involving the addition of buffers, samples, and other reagents to the arrays, the liquids must be spread out across the entire outlined array using a pipette tip. A clean pipette tip is used for each array containing different serum sample to prevent cross-contamination. For buffers and detection cocktails, the same pipette tip can be used. This spreading maximizes the reagent’s exposure to the entire array of printed peptides and improves sensitivity. In addition, a small amount of water must be kept in the hybridization chamber throughout the immunolabeling procedure in order to maintain the humidity required to prevent evaporation of the reagents—especially in steps that require incubations at higher temperatures.
7. In addition to individual patient samples, a system of positive and negative controls is required for each peptide microarray experiment to ensure that the assay is working properly. This system can also be used for data normalization between different microarray experiments. Some options include printing fluorescent-labeled proteins in the arrays that can be detected and compared from experiment to experiment, or in the case

of our facility, the use of positive and negative control patient samples in every experiment. The positive control is a serum pool of severely allergic patients for which the IgE binding patterns are well established. The negative control can be a pooled serum sample of non-allergic, non-atopic patients, or individual non-allergic patient's serum sample.

8. Overnight serum incubation at 4 °C and detection antibody incubation for 4 h at 31 °C are the optimal conditions based on our current peptide library and other experimental conditions. Peptides from different companies, with different levels of purity or printed at different concentrations, may show binding at various levels of intensity. Other factors, such as temperature and humidity levels in the laboratory, may also affect binding diversity and intensity. It is strongly recommended for new facilities to test different printing and immunolabeling conditions and find the conditions that give maximum binding sensitivity without compromising specificity or giving excess signals in the negative controls.
9. EDTA serves to bind any metal ions that may be present in order to prevent the activation of nucleases that will hydrolyze and interfere with the dendrimer signal amplification system.
10. Salmon sperm DNA serves two purposes in this assay: It blocks nonspecific binding of dendrimer molecules to the array, and it protects against nucleases that may be present in the buffer.
11. The Microarray Manager software installed with the NanoPrint60 is used to generate a .gal file that, when opened with the ScanArray Express software, creates a grid on the scanned microarray image and is aligned over the printed spots (Fig. 2b). This grid maps where each array element is spotted, including negative control buffer spots and positive grid control spots. In our facility, we print grid control spots composed of a fluorescent-labeled bovine serum albumin (BSA) on the bottom-right corner of each sub-array. These are helpful with the alignment procedure, and ensure that signals from the correct peptides are digitized and analyzed.
12. For a Gaussian distribution, the MAD is the same as the standard deviation. But the distribution of all the readouts of the blank spots is not necessarily Gaussian, the MAD are more robust estimates of the spread than the standard deviation. Given that the data consists of $x_1, x_2, \dots, x_n$, the MAD is defined as $1.48 \times \text{Median}(|x_i - m|)$, where $m = \text{Median}(x_i)$. The constant 1.48 is used to ensure that if the data's distribution is really Gaussian, then MAD is equal to the standard deviation.

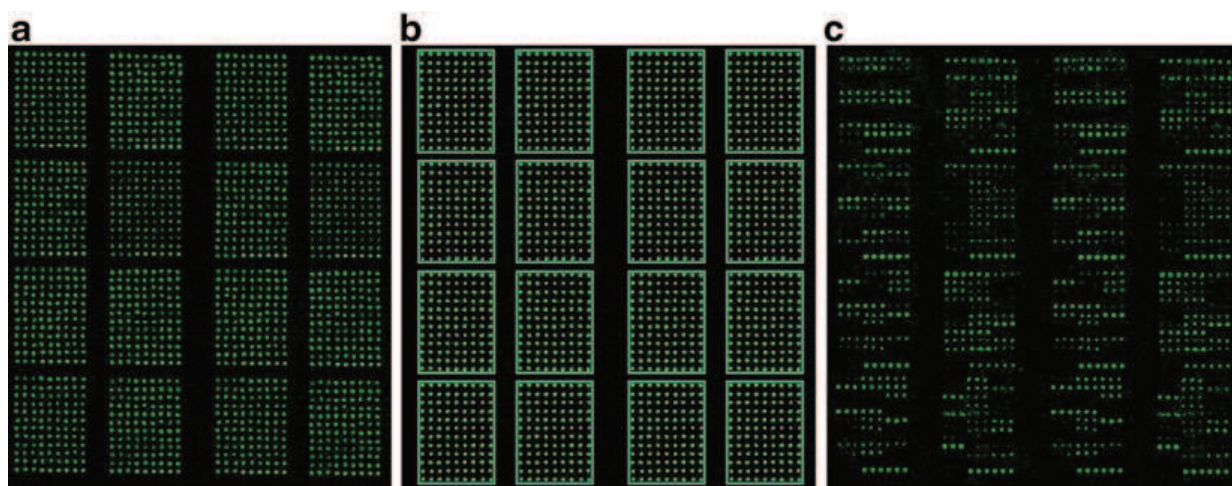


Fig. 2 Scanned images of microarray slides using the ScanArrayGx microarray scanner (PerkinElmer). **(a)** QC scan of the slide after printing to ensure proper printing. In general, spotted peptides are consistent in size and shape, with some minor variations. After washing, all the spots become invisible except the six grid control spots on the *lower right* side of each sub-array. **(b)** A microarray overlaid with the .gal file grid carefully aligned over the spots in order to obtain fluorescence data. **(c)** Final scan of an array immunolabeled with the serum of a severe allergic patient. While binding diversity varies from patient to patient, fluorescent signal from peptides does tend to decrease with lower IgE-level patients, and is blank (with the exception of grid control spots) in negative control samples

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