

IgE Epitope Mapping Using Peptide Microarray Immunoassay

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Abstract

IgE epitope mapping has the potential to become an additional tool for food allergy diagnosis/prognosis and to lead to a better understanding of the pathogenesis and tolerance induction of food allergy. Due to its ability to screen thousands of targets in parallel using small volumes of sample, peptide microarray has greatly facilitated large-scale IgE epitope mapping. In the past 10 years, we have developed and optimized a reliable and sensitive peptide microarray immunoassay, which has been successfully applied for IgE epitope mapping of many food allergens in our lab. Here, we describe the method of performing the peptide microarray immunoassay for IgE epitope mapping. In addition, we have upgraded the microarray platform to measure antibody affinity by adding one additional competition step, which is also described in this chapter.

Key words IgE, Food allergy, Epitope mapping, Peptide microarray , Antibody affinity, Immunoassay

1 Introduction

Food allergy has emerged as an important public health problem in many countries [1]. The most common type of food allergy is mediated by immunoglobulin E (IgE) antibodies through their bindings to specific allergens. The groups of amino acids within allergenic proteins that are bound by IgE antibodies are called IgE-binding epitopes or B-cell epitopes. Identification of IgE-binding epitopes in food allergens will contribute to the design of safe immunotherapeutic agents and a better understanding of the pathogenesis and tolerance induction of food allergy. In addition, studies have suggested a role for sequential IgE-binding epitopes as biomarkers for characterizing various phenotypes of food allergy and may be used for clinical allergy diagnosis and prognosis [2].

Due to its ability to screen thousands of targets in parallel using small volumes of sample, the peptide microarray immunoassay has been applied in our laboratory for large-scale epitope mapping of IgE antibodies [3, 4]. 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 ($r > 0.9$) [4], and detecting IgE binding from diluted serum samples with whole food protein-specific IgE levels as low as 0.068 kU_A/L [5]. Using our peptide microarray, we have successfully mapped the sequential IgE-binding epitopes for a variety of food allergens, such as allergens from peanut/tree nuts, milk, egg, lentil, shrimp, soybean, etc. [6–9], and identified several IgE epitopes as candidate biomarkers for allergy diagnosis/prognosis or tolerance prediction [5, 8].

Using the peptide microarray immunoassay, we are able to measure in one single assay the concentration and diversity of a patient's IgE antibodies toward different allergenic epitopes, but it does not provide information about the affinity of IgE antibodies. Two important steps in the progressive differentiation of antigen-specific B cells are clonal expansion and affinity maturation in which antibody clones with higher affinity expand and further diversify [10]. In allergy, it may lead to IgE antibody clones with higher diversity and affinity. Thus, characterization (i.e., concentration, diversity, and affinity) of antibody clones in the IgE repertoire may reveal a patient's allergic history and/or current status. So far most studies on IgE epitopes focused on the concentration and diversity of IgE antibodies, but not the affinity of IgE antibodies due to the difficulty in measuring antibody affinity.

One method to measure antibody affinity is to use surface plasmon resonance (SPR)-based biosensors, which measure the binding affinity and kinetics of antigen and antibody interaction. Christensen et al. [11] demonstrated that higher affinity IgE binding to Der p 2, as measured using SRP technology, was associated with allergy effector cell degranulation. But this requires specific equipment that is not commonly available in most laboratories. Another method is to use thiocyanate, a chaotropic agent that interferes with non-covalent binding, to remove low affinity IgE bound to an allergen. Using a thiocyanate ELISA elution assay, El-Khouly et al. [12] found a weak correlation between the affinity of peanut-specific IgE to Ara h 2 and the severity of peanut allergy.

Compared with thiocyanate elution, which works by nonspecifically dissociating antigen–antibody complexes, the use of competing proteins to specifically elute IgE antibodies with lower affinity binding to the epitopes of the same protein may be a more relevant indicator of antibody affinity. We therefore included one additional competition step to enable our peptide microarray immunoassay to measure antibody affinity. Using the competitive peptide microarray assay, we were able to show that higher IgE affinity was associated with more severe allergic reactions to milk allergens [8].

In this chapter, we provide the detailed protocol of IgE epitope mapping using the peptide microarray immunoassay. This method is not only useful for epitope mapping of IgE antibodies, but can also be applied to other immunoglobulin subclasses (e.g.,

IgG4) by switching to the corresponding secondary antibody. In addition, we describe the additional competition step that can be added to the regular protocol to measure the affinity of peptide-bound antibodies.

2 Materials

1. Microarray printing slides: SuperEpoxy 2 (SME2) glass slides from ArrayIt. Store the slides under vacuum before printing and use within 6 months of the manufacturing date. The notch on the upper right corner of the slide indicates that samples should be printed on the side facing upward.
2. Peptides: A library of overlapping peptides (*see Note 1*), consisting of 20 amino acids, overlapping by 17 (3-offset), corresponding to the primary sequence of the target protein(s), are commercially synthesized by JPT Peptide Technologies GmbH (*see Note 2*). The synthesized peptides are supplied as lyophilized powder with high purity (QC control by HPLC and mass spectrometry).
3. 384-well, round-bottom, non-sterile assay plates with lids.
4. Ultrasonic bath.
5. Microarray printer: NanoPrint 60 with 2 × 4 Stealth Micro Spotting Pins (SMP3B), controlled by the NanoPrint Microarray Manager Software (ArrayIt).
6. Slide Staintray that can fit up to 10 or 20 slides and hold water to maintain proper humidity. The staintray should be protected from light during immunolabeling.
7. Pap pen.
8. High-throughput slide wash station (ArrayIt) that is equipped with a magnetic stir bar and a slide rack to wash up to 25 slides at the same time. Slides can be washed inside this wash station on a magnetic stir plate.
9. Dimethyl Sulfoxide (DMSO), 99.7 + %, Extra Dry over Molecular Sieve, from Acros.
10. Phosphate-Buffered Saline (PBS): 10× stock solution. 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.
11. Phosphate-Buffered Saline-Tween (PBST): 0.5% Tween-20 in PBS. Add 1 mL of Tween 20 to 2 L of 1× PBS.
12. 1 mM EDTA in PBST: Prepare a 0.5 M stock solution of EDTA by dissolving 186.12 g in 800 mL dH₂O, adjusting pH to 8.0 with NaOH or HCl, and top up to 1 L with dH₂O. Prepare the 1 mM solution by diluting 100 μL of 0.5 M EDTA with PBST to a final volume of 50 mL.

13. 15 mM Tris–HCl buffer, pH 8.0: Prepare 1 M Tris–HCl buffer, pH 8.0 by dissolving 121 g Tris base in 1 L distilled water and adjusting pH to 8.0 with concentrated HCl. Dilute 15 mL of 1 M Tris–HCl buffer, pH 8.0 with 985 mL of distilled water to make 15 mM Tris–HCl buffer, pH 8.0.
14. Protein Printing Buffer (PPB): 2× solution, from ArrayIt. 2× PPB is used to dilute the peptides before printing.
15. Blocking Buffer: 1% human serum albumin (HSA) in PBST. It is used to block all unbound sites on the slide surface prior to incubation with serum samples, and as the diluent for both serum and detection antibodies.
16. Dendrimer Buffer: UltraAmp Binding Buffer II from Genisphere.
17. A serum pool of several patients allergic to the specific food/allergen is served as positive control and a pooled serum sample of non-allergic, non-atopic patients, or individual non-allergic patient's serum sample is served as negative control (*see Note 3*).
18. Competition mixture: The competition mixture is derived from the same food proteins that the printed peptide sequences are derived. For example, if the printed peptides are based on the amino acid sequence of milk allergens, use the same milk allergens for competition. Prepare a stock solution of the proteins in PBS, then dilute to the appropriate concentration using blocking buffer and mix with other proteins.
19. Anti-Human IgE cocktail containing several Biotin conjugated monoclonal mouse Anti-Human IgE antibodies in blocking buffer (*see Note 4*). The antibody cocktail must be prepared freshly right before use. A few antibodies that we have been using are Biotin conjugated monoclonal Mouse Anti-Human IgE from BD Biosciences, Biotin conjugated monoclonal Mouse Anti-Human IgE (ϵ -chain specific) from Molecular Probes by Life Technologies.
20. Dendrimer signal amplification system: UltraAmp Anti-Biotin oyster 550, from Genisphere.
21. Salmon Sperm DNA.
22. ScanArray Gx Microarray Scanner from PerkinElmer.

3 Methods

3.1 *Printing and Preprocessing of Peptide Microarray Slides*

1. Preparation of printing plate: The lyophilized peptides are resuspended in DMSO at 1 mg/mL, diluted 1/2 in 2× protein printing buffer (PPB) (*see Note 5*), and transferred (10–15 μ L/well) into the printing plate with lid. The printing plate is centrifuged at 300 rcf for 5 min to remove any bubbles that may

affect printing, and stored in a sealed plastic bag at -80°C until use. The printing plate can be used for up to five printings.

2. Before peptide printing, the printing plate containing peptides in printing buffer is left at room temperature for 10–15 min, placed on a pedestal in an ultrasonic bath, sonicated for 60 s, and centrifuged at 300 rcf for 3 min at room temperature.
3. Peptides are printed from the printing plate onto the SME2 slides using the NanoPrint 60 microarray printer loaded with the Stealth Micro Spotting Pins (SMP3B) (*see Note 6*). The peptides are printed in two sets of triplicate spots. In addition to peptides, 20% of the printed spots are “blanks” composed of 50% v/v DMSO in PPB as negative control for background normalization. It is recommended to print some fluorescent-labeled protein at specific location to facilitate grid alignment. Other proteins, such as different concentrations of purified human IgE, may be printed to serve as positive controls. In order to save slide space and serum volume used for immunolabeling, two arrays can be printed on a single slide.
4. During printing, the humidity is maintained at 55–65% to avoid sample evaporation. Once completed, humidity is turned off and slides are dried overnight in the printer to ensure peptide binding to the slide substrate.
5. Following overnight drying, take out the slides from the microarray printer and scan the printed slides using the microarray scanner to monitor the quality of printed spots. Any slide with missing spots should be discarded or marked.
6. Use pap pen to make hydrophobic barrier around the printed area to create a rectangular incubation area for immunolabeling. Make sure there is enough space between the barrier and the printed spots to allow for proper hybridization. The two arrays on each slide can be separated using pap pen. Leave the slides at room temperature for at least 2 h to let the ink dry.
7. Wash the slides twice, each for 20 min, with PBST and 0.05× PBS respectively, in the high-throughput wash station with stirring to remove unbound peptides. This prewash can reduce nonspecific binding and background noise. Slides are then dried by centrifugation at 130 rcf for 3 min. Following centrifugation, the slides can be immunolabeled right away or stored in a vacuum container for up to 3 weeks.

3.2 Immunolabeling of Microarray Slides

All the incubation steps in immunolabeling are performed in a slide staintray/humidity chamber protected from light on a rotating platform with gentle agitation/rotation. It is very important to add water into the humidity chamber to maintain proper humidity and keep the slide covered with solutions/buffers at all times; never let the slide dry during immunolabeling. All solutions/buffers should

be removed by aspiration at the corner of each array (without touching the printed spots) unless otherwise stated.

3.2.1 Blocking

1. Place the slides on the hybridization chamber, and add 250 μL of blocking buffer onto each array. Incubate for 1 h at 31 °C with gentle rotation.
2. Carefully remove the blocking buffer by aspiration.

3.2.2 Serum Incubation

1. Patient serum samples (*see Note 3*), diluted 1:5 in blocking buffer, are added to the arrays (250 μL /array) and gently rotated overnight at 4 °C (*see Note 7*).
2. Following overnight incubation, serum is removed by gently tapping the slides on tissue paper. If there are more than two arrays per slide, avoid contamination between arrays.
3. The slides are transferred onto a slide rack, dipped two times in PBST, and washed in the high-throughput wash station containing fresh PBST 2 $\times$ 5 min at room temperature.
4. Place the slides back into the hybridization chamber and the remaining PBST is removed by aspiration.

If the purpose of the experiment is to measure the amount of IgE antibodies to each peptide, skip Subheading 3.2.3 and continue with Subheading 3.2.4. If the purpose of the experiment is to measure the affinity of peptide specific IgE antibody, continue with Subheading 3.2.3.

3.2.3 Competition with Allergen

The conditions (e.g., temperature, competitor concentration, and incubation time) for performing the competition vary depending on the patient population and the allergens, and should be optimized before applying onto individual serum samples. We use a scatter plot to evaluate the effect of competition by plotting the standard conditions (standard protocol without the competition step) vs. the competition, or competition with blocking buffer which contains human serum albumin as a negative control vs. competition with the specific allergen. When optimizing using a serum pool, which contains a mixture of high and low affinity antibodies, the conditions resulting in more scattered points in the scatter plots are usually selected as it reflects the different affinity of IgE antibodies to different epitopes (*see Note 8*).

As an example, Fig. 1 compares different competition temperatures using a serum pool of five patients highly sensitive to milk. Competition at 31 °C seems to be too harsh because IgE binding to most peptides is competed off. Competition at 16 °C is selected because the points are more scattered.

1. The competition mixture is derived from the same food proteins that the printed peptide sequences are derived and is added to the arrays (250 μL /array) and gently rotated for 1 h at 16 °C.

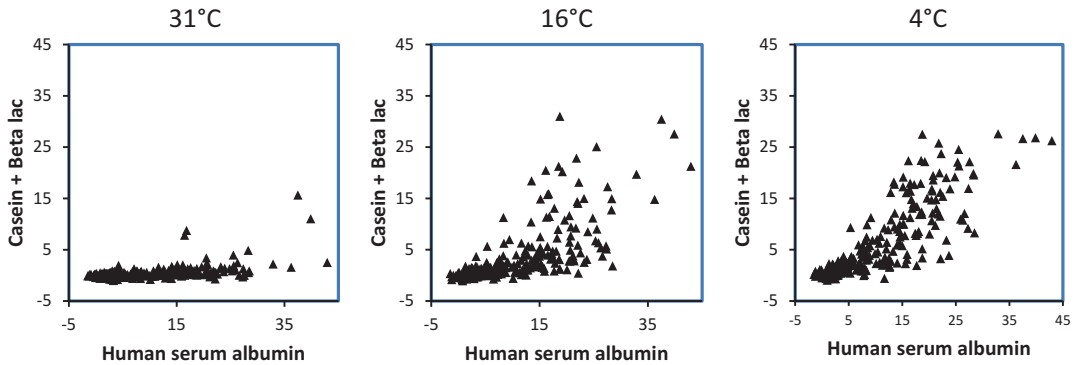


Fig. 1 Scatter plot comparing IgE binding between competition with a negative control (blocking buffer containing human serum albumin, x-axis) and competition with a mixture of 2 mg/mL caseins and 3 mg/mL beta-lactoglobulin (y-axis) at different temperatures for 4 h. Each spot represents IgE binding (represented as a z score) to 1 peptide of milk allergens

2. Gently tap the slides on tissue paper to remove the competition mixture, then wash the slides 2×5 min in the high-throughput wash station containing fresh PBST at room temperature.
3. Place the slides back into the hybridization chamber and the remaining PBST is removed by aspiration.

3.2.4 Secondary Antibody Incubation

1. Prepare the secondary antibody, which is a cocktail of several Biotin-conjugated monoclonal mouse Anti-Human IgE antibodies right before the incubation. The antibodies are each diluted 1:250 in blocking buffer. The concentration of each antibody needs to be optimized using a serial dilution of the antibody and choose the dilution that gives the strongest signal versus low background.
2. Add 250 μL of the antibody cocktail onto each array, and incubate for 4 h. If possible, incubate at a slightly higher temperature (e.g., 31 $^{\circ}\text{C}$ for 4 h) to increase sensitivity (*see Note 7*).
3. Following incubation, quickly wash the slides (remain in the humidity chamber) with PBST from a squeeze bottle and remove the remaining PBST by aspiration. Add 300 μL of 1 mM EDTA in PBST to each array and leave on a rotating platform for 5 min at room temperature.
4. Quickly wash the slides again with PBST from a squeeze bottle. Remove the remaining PBST by aspiration.

3.2.5 Dendrimer Amplification

This step is to amplify the signal using dendrimers to increase the sensitivity of the assay.

1. Add 200 μL of Dendrimer Buffer onto each array, and leave for 1 min before removing by aspiration.

2. Prepare the dendrimer cocktail (200 μL /array: 12 μL anti-Biotin dendrimer, 4 μL salmon sperm DNA, and 184 μL dendrimer buffer) right before adding them onto the slide. If possible, incubate at a slightly higher temperature (e.g., 31 $^{\circ}\text{C}$ for 4 h) to increase sensitivity (*see* **Note 7**).
3. Following incubation, quickly wash the slides (in the humidity chamber) with PBST from a squeeze bottle. Transfer the slides onto a slide rack. Dip ten times in 15 mM Tris-HCl and dry the slides by centrifugation. Dip five times in 0.1 $\times$ PBS and dry by centrifugation. Dip five times in 0.05 $\times$ PBS and dry again (*see* **Note 9**). Scan the slides or store in vacuum.

3.3 Slide Scanning

1. Scan the immunolabeled slides using a ScanArray[®]Gx microarray scanner. Save the scanned images as TIF files.
2. Align the image with a gal file that contains information on sample location and ID and is generated by the NanoPrint Microarray Manager Software (ArrayIt) for each printing. The fluorescent signals of all spots are digitized with ScanArray Express software (Perkin Elmer) and exported as comma-delimited text (.csv) files, which contains mean and median fluorescent signal (expressed as digital fluorescence units [DFUs]) of each spot and its local background.

3.4 Data Analysis

The following calculation can be performed using R programming language (<http://www.r-project.org/>), or other software that does not require programming, such as Microsoft Excel.

1. Calculate the readout (S_i) for each spot, including the replicates for peptides and blanks (PPB + DMSO), by using the equation below.

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

2. Transform the S_i for each spot into a Z score (Z_i) by using the equation below.

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

Median (S_{Blank}) and MAD (S_{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.

An index Z value of each peptide is generated by taking the Median of Z scores of the 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 the signal is significantly above the background with p value less than 0.003.

3. (Optional) In order to eliminate nonspecific background noise, single-positive peptides with negative binding by four adjacent peptides (with overlapping amino acid sequence) are filtered out by replacement with the median of itself and the four adjacent peptides.
4. Further data analysis (e.g., Wilcoxon test, clustering) and presentation (e.g., Heat map) can be performed using R programming or other software such as Microsoft Excel, GraphPad Prism, and TIGR Multiexperiment Viewer (TMeV).

4 Notes

1. The use of 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 solid phase bound peptides may not always be exposed and available for binding with IgE antibodies. By using overlapping probes, we increase the chances of detecting all epitopes.
2. Currently, there are quite a lot of companies in the market providing custom peptide synthesis service. We have also used the peptide synthesized by Chinapeptides Co., Ltd. (Shanghai, China) who supplies lyophilized peptide with purity >90%.
3. In addition to individual patient samples being tested, a positive and a negative control are required to run together with the serum samples being tested in each peptide microarray experiment to ensure that the assay is working properly and for data normalization. A serum pool of several patients allergic to the specific food/allergen can serve as a positive control and a pooled serum sample of nonallergic, non-atopic patients, or individual non-allergic patient's serum sample can serve as a negative control. These control serum pools are very important as they can be used for optimization of the experimental conditions, validation of antibodies, and controls run in each experiment for data normalization.
4. We recommend using a cocktail of more than one monoclonal antibody (higher specificity than polyclonal antibody), and the specificity of the antibody needs to be validated before incorporating them into the cocktail.

5. Sometimes, some peptides tend to form smaller spots after printing. This may be improved by adding Sarkosyl. We have tried to improve spot morphology using 0.02% of Sarkosyl.
6. We have tested the printing consistency of the SMP3B printing pins by printing fluorescent labeled proteins without reloading and found that the pin can deposit the same amount of samples and print at least 250 consistent spots. Therefore, the pins are usually used to print no more than 200 spots from each sample loading. In addition, the first two printed spots after each sample loading are usually very large and should not be used. We usually place two microscope glass slides on the first two printing positions and then discard them after printing. 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. In addition, it is important to clean the pins properly between loading different peptides to prevent cross-contamination. This can be done by washing the pins for 2 min between each loading of peptides in the sonicating bath station (filled with 10% ethanol) in the microarray printer.
7. The current experimental protocol (e.g., overnight serum incubation at 4 °C and secondary antibody incubation for 4 h at 31 °C) has been optimized based on our current peptide library and other experimental conditions, and may be affected by many factors, such as peptide purity, temperature and humidity in the lab, secondary antibody batch, etc. When setting up the peptide microarray facility, it is recommended to test different printing and immunolabeling conditions using the positive and negative control sera and find the optimal conditions that give maximum binding without compromising specificity. If any of the factors is changed, it is better to test the relevant conditions again to ensure optimal conditions.
8. The optimization of the competition condition is the key step in the measurement of antibody affinity using the microarray. The optimal conditions are the conditions that can differentiate antibodies with low and high affinity: Low affinity antibodies are competed off while high affinity antibodies remain bound. As noted above, this will result in more scattered points in scatter lots.
9. The final wash aims to remove unbound dendrimer molecules and reduce excess salt deposits on the array surface that will give high background noise. It is not recommended to wash the slides with water because this will remove bound dendrimer.

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