

Protein Microarray-Based IgE Immunoassay for Allergy Diagnosis

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Abstract

Protein microarray is a miniaturized multi-analyte, solid-phased immunoassay where thousands of immobilized individual protein spots on a microscopic slide bind are bound to specific antibodies (immunoglobulins) from serum samples, which are then detected by fluorescent labeling. The image processing and pattern recognition are then quantitatively analyzed using advanced algorithms. Here, we describe the use of an in-house-produced complex protein microarray containing extracts and pure proteins that has been probed with antibodies present in the horse sera and detection by fluorophore-conjugated antibody and data analysis. The flexibility of the number and types of proteins that can be printed on the microarray allows different set of specific IgE immunoassay analysis to be carried out.

Key words Protein microarray, Component-resolved diagnostics, Microarray-based allergy testing, Horse sera, IgE-binding assay, Partial Least Squares Discriminant Analysis (PLSDA), Variable influence on the projection (VIP)

1 Introduction

Protein microarray is a miniaturized multi-analyte, solid-phase immunoassay where proteins are immobilized or “spotted” onto a solid surface of a size of microscopic slide and hybridized or probed against microliters of serum samples. Any specifically bound immunoglobulins (Ig) from sera are then labeled with fluorophore-conjugated antibodies and detected under standard conditions.

Protein arrays of pure proteins and extracts have shown to be reproducible, sensitive, and very flexible regarding the different types of Igs that can be monitored simultaneously. Generally, the results are comparable to other standard laboratory methods such as UniCAP, ELISA, and immunoblot tests but with an improved efficiency in terms of quantities of the sample used.

Previously in our lab, we have developed protein microarray-based immunoassays for detection of food and environmental

allergens for humans [1–3] and more recently allergenic components from insect saliva used in equine allergy have been added [4].

The current array contains ca. 240 complex extracts and 120 pure proteins from food (fruit, dairy, seeds, fish, mollusks), environment (epithelium, pollen, fungi, and insects including recombinant *Culicoides* allergens) that are commercially available or in-house-produced. In these complex arrays, extracts and pure proteins are represented and a complex series of mathematical modeling procedures (discussed in [4]) has been developed and used to classify and guide the analysis.

The protein microarray used in this chapter was developed by spotting normalized purified protein solutions and extracts of interest onto 16-pad nitrocellulose FAST slide format using Marathon microarrayer, which was carried out by Arrayjet Ltd, Roslin, Scotland. The resulting 16-pad nitrocellulose FAST slide contains 12,288 spots per slide and each pad consists of 384 proteins, extracts, and controls spotted in duplicate at similar position (see Fig. 1c). Details of methodology for printing protein arrays will not be covered in this chapter.

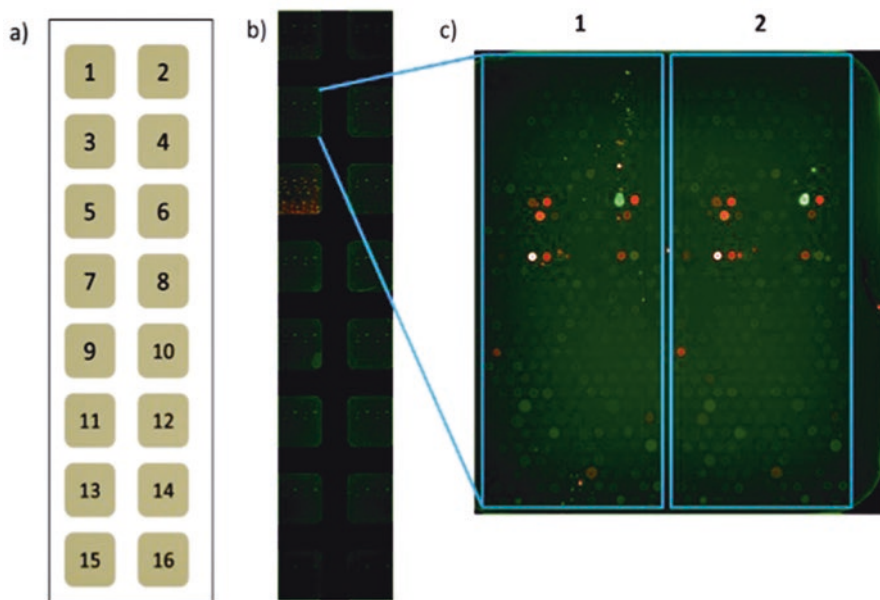


Fig. 1 (a) A diagram showing the 16-pad nitrocellulose FAST slide array that is printed with protein extracts and purified proteins from food, environment (including recombinant *Culicoides* allergens), and positive controls. The in-house assembled protein microarray slide contains 16 pads with a total of 12,288 proteins per slide printed in duplicate. (b) An image of a microarray after probing with 15 serum samples and a control, and detecting with DyLight647-labeled antibodies and visualized using GenePix 4000B scanner. (c) Pad '3' of the scanned microarray of a control (2% BSA in 0.2% PBST only) is shown with 384 protein spots printed in duplicate at similar positions in box '1' and box '2'. The array pad contains positive controls such as Cy5 and Cy3 conjugated proteins to help identify arrays while gridding the spotted antigens

2 Materials

2.1 Equipments

1. Printed 16-pad nitrocellulose slide arrays (*see Note 1*).
2. Corning® 5 slide Holder.
3. Mini slide boxes (25 slides).
4. Proplate slide module (Grace Bio-Labs, Inc.).
5. Proplate® Multi-Well Chambers with silicone gasket (Grace Bio-Labs, Inc.).
6. Proplate® adhesive seal strips with seal strip applicator (Grace Biolabs, Inc.).
7. Mistral 3000i centrifuge (4-place wind-shielded swing-out rotor (43124–708) with 2 × 96-well plate holders).
8. Mini Hybridization Oven with a rotator.
9. Thermo-Hypro 20 Hybridization Unit.
10. ELx50 Washer (BioTek Instruments, Inc., Winooski, VT, USA).
11. GenePix 4000B scanner (Molecular Devices, USA).

2.2 Buffers and Solutions (See Note 2)

1. 10 mM phosphate buffered saline, pH 7.4 (1× PBS): dissolve 8 g NaCl, 0.2 g KCl, 1.44 g Na₂HPO₄ and 0.24 g of KH₂PO₄ in 800 ml deionized water, adjust to pH 7.4 with 2 N HCl and adjust volume to 1 L with deionized water.
2. 0.2% PBST: 0.2% (w/v) Tween 20 in 1× PBS.
3. 1% BSA: 1% (w/v) bovine serum albumin, low endotoxin, pH 7, ≥ 98% (BSA) in 0.2% PBST.
4. 2% BSA: 2% (w/v) BSA in 0.2% PBST.
5. 3% BSA: 3% (w/v) BSA in 0.2% PBST.
6. Wash buffer: 0.05% (w/v) Tween 20 in 1× PBS.

2.3 Sample

1. Horse sera (kept at −80 °C when not in use) (*see Note 3*).

2.4 Antibodies

1. Detection antibody: mouse anti-horse IgE mAb (1 mg/ml) [7].
2. Labeled secondary antibody: Dylight 649-labeled rabbit anti-mouse IgG Ab (Dylight 649, Rockland Inc., USA) (1 mg/ml).

2.5 Software

1. GenePix Pro software v6.0.1.27 (Axon Instruments).
2. Microsoft Excel software (Microsoft, USA).
3. Matlab (Version 9, The Mathworks Inc., USA).
4. Excellink toolbox (Mathworks) and Dataset Object (Version 5.0, Eigen-vector Research Inc., USA).

3 Methods

3.1 Sera Sample Preparation

1. Aliquot sera samples from horse patients/controls into smaller volume (100 μ l/tube) and store aliquots at -80°C until further use (*see* **Note 3**).
2. Thaw and keep sera samples on ice when in use.

3.2 Probing Protein Microarray

1. Label printed 16-pad array slides for identification.
2. Block the printed 16-pad array slides with 3% BSA. Place a maximum of three slides in each Corning® 5 slide holder and fill the slide holder with approximately 25 ml of 3% BSA in PBS without air bubbles.
3. Close the cap of the slide holder, sealed it with parafilm, and covered it with aluminum foil to prevent light exposure.
4. Incubate the slides for 3 h at 37°C with rotation in a mini hybridizing oven and in the dark.
5. Wash the slides with 0.05% PBST three times for 2 min each. This is followed by 5 times washing with deionized water for 1 min each. Place slides in a slide holder box and place the box onto the 96-well plate holder. Dry slides by centrifugation (Mistral 3000i, rotor 43,124-708, with a 96-well holder) at $300 \times g$ for 10 min at a room temperature (*see* **Note 4**).

3.3 Hybridization

1. Assemble the Proplate slide module (Grace Bio-Labs) by aligning edges of slides with the edges of upper structure (*see* **Note 5**).
2. Press gently on back side of the slide to adhere the slide to the gasket.
3. Slide clips until both ends are flush with the assembly. Make sure that the slide “star” position is at frame number 15.
4. Wet the 16-pad array membrane with 150 μ l of 0.2% PBST per well.
5. Dilute horse sera in 1:2 with 2% BSA in a flow cabinet (*see* **Note 6**).
6. Aspirate 0.2% PBST from wells using multi-channel pipette without touching the surface of the slide.
7. Add 100 μ l of diluted serum sample to each well. For each slide, dedicate a well (one of 16 pads) as a control by adding 100 μ l of 2% BSA only.
8. Seal the Proplate slide module with Proplate adhesive seal strips (*see* **Note 7**).
9. Incubate slides in Proplate assembly in Thermo Hypro 20 hybridizer at 37°C for 2 h with vibration (set to AVS 3).

3.4 Detection

1. Wash slides in Proplate assembly three times with 0.05% PBST using ELx50 Washer (*see Note 8*).
2. Dilute detection antibody, mouse anti-horse IgE mAb, in 1:400 in 1% BSA.
3. Add 100 μ l of the diluted detection antibody to each well and incubate in Thermo Hypro 20 hybridizer at 37 °C for 1 h with vibration (set to AVS 3).
4. Wash slides in Proplate assembly three times with 0.05% PBST on ELx50 Washer.
5. Dilute labeled secondary antibody, DyLight 649 conjugated anti-Mouse IgG1, in 1:400 in 1% BSA.
6. Wash slides in Proplate assembly three times with 0.05% PBST and three times with deionized water using ELx50 Washer.
7. Carefully slide clips off and transfer the slide to a slide box with some tissue papers at the bottom.
8. Centrifuge slide at $300 \times g$ (Mistral 3000i, rotor 43,124-708, with a 96-well holder) for 10 min at a room temperature to dry the slide (*see Note 4*).
9. Scan the slide on a dedicated scanner (GenePix 4000B) using the PMT parameter settings as 440 (645 nm) and 310 (532 nm). Save the image as tiff file.

3.5 Data Analysis

1. Create a gal (GenePixArrayList) file to indicate the position of each protein spotted on the array using GenePix Pro 6.1 software (Molecular Devices Corporation).
2. Grid the tiff image of scanned slide with the gal file using GenePix Pro 6.1 software (*see Note 9*).
3. Analyze the image to get the fluorescence intensity readings for each spot in the GenePix Result (gpr) file.
4. Subtract readings of a local background for each spot (around the diameter of the spot) from the fluorescence intensity reading of the spot.
5. In the gpr file, the fluorescence intensity of each spot is presented and annotated in a table where the mean, median standard error, and local background readings for each spot are tabulated and used for further calculation purposes.
6. Export gpr file into Microsoft Excel.
7. Average duplicate spot readings for each pad (both samples and a control).
8. Subtract averaged spot readings of control from averaged spot readings of samples to eliminate non-specific binding and inherent autofluorescence of some proteins using Visual Basic macros in-built into Microsoft Excel software (Microsoft, USA).

9. Comply normalized sera sample data and transfer them from Microsoft Excel to Matlab (Version Matlab (version 7.1 (R14SP3), The Mathworks Inc., USA) using Excel link tool-box (Mathworks) and the Dataset Object (Version 5.0, Eigenvector Research Inc., USA).
10. Perform Partial Least Squares regression (PLS) to establish the multivariate link between the array data sets and the results from standard IgE immunoassay (such as CAP) results.
11. Perform Partial Least Square Discrimination analysis (PLSDA) based on Partial Least Squares regression (PLS) model [5, 6] to construct predictive models (*see* **Note 10**). Use the threshold of >1 and scale the means of the data to unit standard deviation by auto-scale.
12. Employ internal cross-validation to assess the number of latent variables (data trends) to build model with minimal predictive error.
13. Confirm all the classification results by cross-validation where random subsets of the data are left out.
14. Build regression model with increasing number of latent variables and establish the prediction error for the left out samples for each model. The influence of individual features may be captured with variable influence on the projection (VIP) derived from the PLS coefficients for the optimal set of components from the PLS model (*see* Fig. 2).
15. Repeat **step 14** until all samples have been left-out once and establish an average prediction/classification error per number of latent variables. The result is an estimation of the most appropriate number of latent variables (with the lowest error) as well as an estimation of the prediction/classification error to be expected when applying the model to new data (*see* Fig. 3).

4 Notes

1. Printed microarray slides can be kept at room temperature for a long time but need to be stored in a dark and dry area.
2. It is better to prepare fresh buffers every time before the analysis. Buffers such as PBST may be kept at 4 °C for 7 days and 1× PBS may be kept at room temperature for a long time however, BSA in PBST has to be freshly prepared prior to the analysis.
3. To avoid repeated freeze–thawing of the sera used, aliquot sera into smaller quantities and store the aliquots at –80 °C. Keep thawed sera on ice when in use.

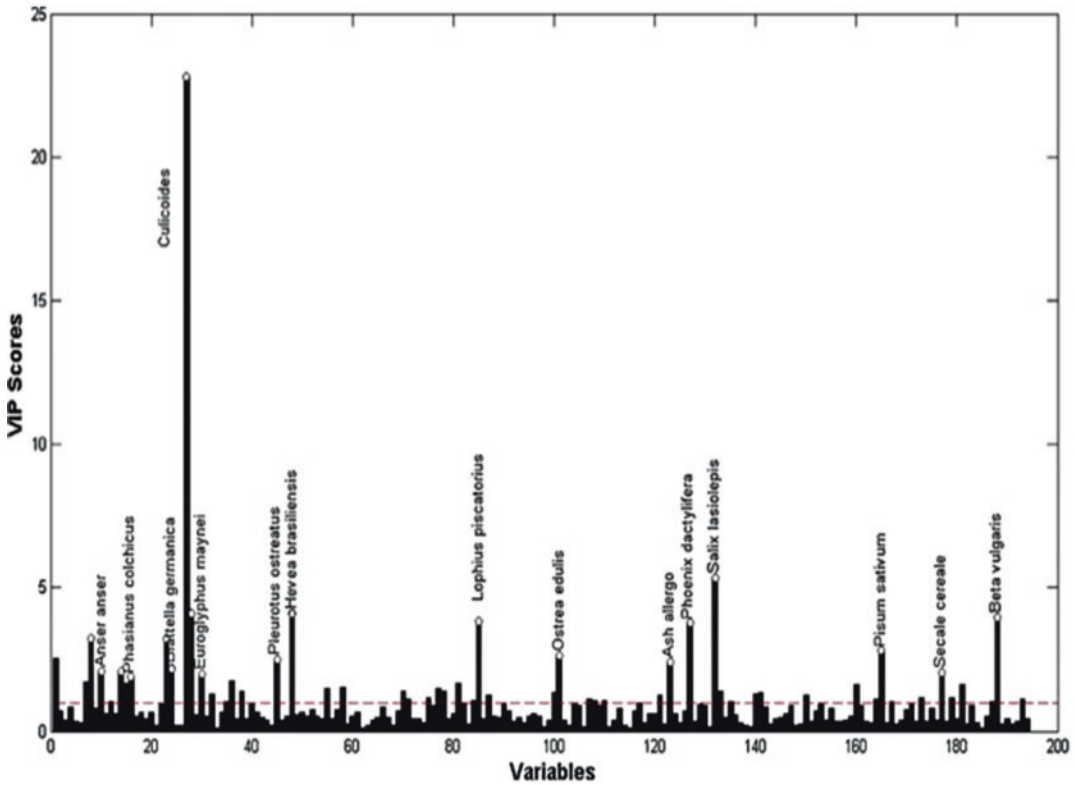


Fig. 2 An example of analyzed comparative result by determining the variable influence on projection (VIP) from insect-bite hypersensitive (IBH)-affected and healthy horses for each protein and extract. VIP is calculated by partial least square discrimination analysis software (PLSDA) from 35 insect-bite hypersensitive (IBH) affected and 37 healthy control horses using 200 averaged variables (extracts and pure proteins). The mean variable of *Culicoides nubeculosus* and *C. obsoletus* (extract and pure proteins) was the major variable identified as important for this classification. Some minor contributing variables are also shown. Graph is derived from a published data [4]

4. Tissues are placed at the base of the slide holder to quicken the drying process. Do not let the slide air-dry and the slide should be taken out quickly in the flat slide holder box for drying.
5. Place microscope slide (array side down) over silicone gasket, aligning edges with the edges of the upper structure and make sure that the slide properly adhere to the gasket. Improper assembly may cause the glass slide to be chipped or broken.
6. All works that involve the use of serum need to be performed in a flow cabinet. If human sera are used, please ensure that the preparation is performed in a designated area for human sample. Use of disposable gloves is mandatory. Any spillage should be wiped with Virkon and disposed in the clinical waste.
7. Proplate-assembled microarray slide need to be properly sealed between the wells. The solution tends to vaporize during the

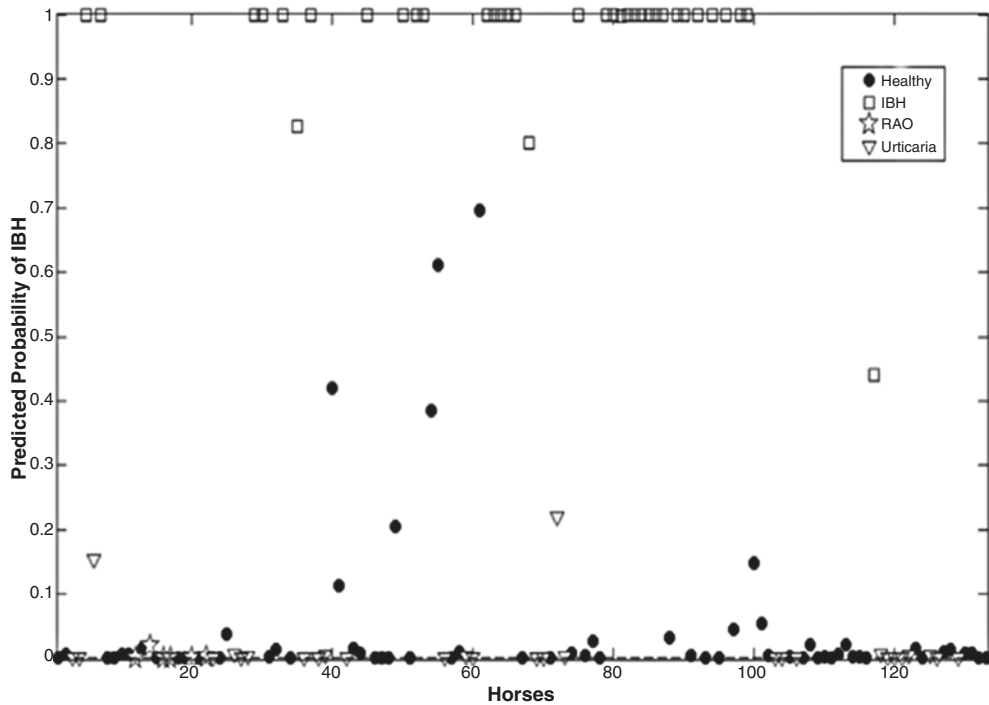


Fig. 3 Prediction probability of belonging to the IBH group of horses. Using the calibration parameters shown in Fig. 2, the predicted probability of each horse to belong to the IBH affected group ($n = 37$) when compared to control horses (healthy, recurrent airway obstruction (RAO) and urticaria; $n = 98$) is shown. The plot graph showed that 36 out of 37 IBH-affected horses had $>70\%$ probability of belonging to the IBH group, estimating that they are to be diagnosed as IBH. Image is derived from a published data [4]

hybridization process so improper sealing may cause the plate to dry. Avoid using parafilm.

8. For washing step, it is recommended to use an automated plate washer. ELx50 Washer used in our lab is an automated plate washer with 16-channel manifold for dual dispensing and aspirating solution that ensure a thorough washing of the array with low residuals. However, if the plate washer is not available, the washing step can be performed manually (but with a risk of remnant residuals).
9. The software to create the gal file that link the spot position with its protein identity is provided with the GenePix scanner but ultimately can also be created as tab delimited file in Microsoft Excel. Grid may need to be adjusted manually to fit the spot on the tiff image of the scanned slide.
10. PLSDA is suited for the analysis of protein microarray due to (a) its ability to handle data in which variables far out-number the number of samples and (b) in which features are correlated (multicollinearity).

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