



Use of Humanized Fluorescent Reporter Cell Line RBL NFAT-DsRed for the Detection of Allergen-Specific IgE in Patient Sera Using Allergen Microarrays

Marina Kalli, Marcos J. C. Alcocer, Andrew J. Blok, and Franco H. Falcone

Abstract

The presence of allergen-specific IgE (sIgE) in human sera can be determined by measuring the binding of sIgE to solid phase-bound preparations containing the allergens to be tested. These can be complex extracts, purified or recombinant allergens, or peptides. Older methods, such as the IgE CAP test, only allow sIgE measurements to multiple allergens in individual measurements. Newer technologies such as the ImmunoCAP® ISAC test allows semiquantitative testing of sIgE to over a hundred allergens on a protein array. Allergen arrays have higher numerical power, allowing testing to many allergens at the same time, using only a small amount of serum. We have previously demonstrated how allergen arrays can be used in combination with purified peripheral blood basophils, introducing a clinically relevant readout. Here, we describe a protocol and materials that allow the testing of sIgE with multiple allergens in array format, using a humanized fluorescent IgE reporter system (RBL NFAT-DsRed).

Key words Fluorescent reporter cell line, RBL, IgE, Allergen, Microarrays, NFAT-DsRed

1 Introduction

The introduction of allergen arrays and component-resolved diagnostics (CRD) is widely considered a milestone in the improvement of diagnostic techniques for IgE-mediated allergy [1]. CRDs take advantage of the ability to assess the binding of allergen-specific IgE (sIgE) to a large number of allergens (purified or recombinant proteins, peptides) printed in array (multiplex) format on suitable surfaces. CRD now plays an increasingly important role in assessing IgE-mediated cross-reactivities in food allergy, hymenoptera venom allergy, and pollinosis.

CRD in multiplex format, in particular when used for diagnosing IgE-mediated food allergy, is able to avoid some issues associated with the more established diagnostic methods, such as oral food challenge (OFC), considered the gold standard in its double-blind placebo-controlled food challenge form (DBPCFC), as well

as skin prick testing (SPT). CRDs can enable the distinction between primary sensitizations to “true” allergens and “mere” IgE cross-reactivities [2]. To some extent, CRDs are also thought to enable prediction of the severity of reactions and potential for dangerous anaphylactic reactions. This is because, in the case of food allergens, sensitizing proteins/epitopes, which are not destroyed by heat or are resistant to digestive enzymes, are more likely to cause symptoms than heat-labile, easily digested proteins [3]. This information becomes assessable through the use of CRD, where sIgE to individual molecules, rather than using complex extracts, is measured. While this would a priori appear to tilt the balance in favor of only using purified or recombinant allergens for diagnosis, some arguments can be made in support of the use of complex extracts. For example, extracts can be useful in cases where the sensitizing allergens may not yet be known or are not yet available in recombinant form, as in recently more globally introduced food sources, such as goji berries [4], tapioca [5], or even edible insects [6]. Furthermore, the safety coverage achieved by using only a few selected major allergens for assessment, bearing in mind the size of the genomes of commonly used food crops such as wheat (*Triticum aestivum*; 14.5 Gbp), soy (*Glycine max*; 1.115 Gbp) [7], or canola [8] (*Brassica napus*; 1.13 Gbp) [9], can be questioned. However, using complex extracts, in addition to the known problematic issues such as stability or difficult standardization, potentially brings back one of the issues already solvable by CRD, the presence of cross-reactive carbohydrate determinants (CCDs) in food extracts. These are carbohydrate epitopes characterized by their ability to bind IgE, but inability to cause clinical symptoms [10]. Therefore, to take full advantage of using complex extracts as an additional way of assessing sensitization to food, a method introducing a biologically (i.e., ultimately clinically) relevant readout is desirable, as this can distinguish between unproductive IgE binding (not leading to cellular activation, therefore clinically irrelevant) and cross-linking sIgE/allergen combinations.

Our group has shown proof of principle that allergen arrays can be combined with live peripheral blood basophils for a biologically relevant assessment of sensitization to food or any other allergen [11]. However, due to the cost and other difficulties with purifying sufficient amounts of basophils from peripheral blood (see Chapter 2) of patients, this solution is unlikely to find widespread use in a clinical facility. Therefore, using a low-cost reporter cell line, which could be grown in practically unlimited amounts and is able to indicate FcεRI-mediated activation by allergens, would make this technology more amenable to a widespread use. However, no detailed protocols for the use of IgE reporter cell lines with allergen arrays are available. Critical parameters in this technology, which combines protein arrays with living cells, are the chemical

structure of the surface used for printing the allergen arrays and details of the protocol, in order to avoid cell toxicity during incubation and cell losses during the washing steps.

Here, we present a protocol, which describes how sensitization to food can be assessed using extracts, purified or recombinant allergens, printed onto allergen arrays, in combination with the recently developed, humanized fluorescent IgE reporter cell line, NFAT-DsRed [12, 13].

2 Materials

2.1 Reagents

1. RBL medium: Eagle's Minimum Essential Medium (EMEM), 10% v/v heat-inactivated fetal bovine serum, 100 U/mL penicillin, 100 µg/mL streptomycin, and 2 mM L-glutamine. Store at 4 °C.
2. RBL-703/21 NFATp-DsRed-Express2 cell line, available from the authors on the basis of an MTA.
3. G418 sulfate (Geneticin).
4. Blasticidin S (for NFAT-DsRed cells).
5. DPBS without Ca²⁺ or Mg²⁺.
6. Trypsin–EDTA or cell scrapers.
7. Patient sera.
8. Allergens.
9. Anti-human IgE.
10. 0.05% (v/v) Tween 20.
11. 1 mg/mL bovine serum albumin (BSA).
12. 10 µg/mL Fibronectin solution in DPBS.

2.2 Supplies

1. Graduated pipettes and pipette tips.
2. 25 cm²/75 cm² cell culture flasks.
3. 50 mL centrifuge tubes.
4. Clear six-well tissue culture-treated plates.
5. 35 mm culture dishes.
6. 16-well ProPlate® Multi-well Chambers (Grace Bio Labs, US, Cat. No 244864).
7. ProPlate® Clips (Grace Bio Labs, US, Cat. No 204830).
8. Amine-coated slides (Surmodics, US, Cat. No. DN01-0025).
9. Parafilm M.

2.3 Equipment

1. Pipettors for graduated pipettes/tips.
2. Water bath.
3. Class II microbiological safety cabinet.

4. Cell culture incubator: humidified incubator set to 37 °C and 5% CO₂.
5. Hemocytometer.
6. Centrifuge capable of holding 50 mL tubes.
7. GenePix[®] 4000B Microarray Scanner (Molecular Devices, US) or a comparable suitable microarray scanner.

3 Methods

3.1 General Cell Culture

Work in a Class II microbiological safety cabinet using sterile techniques.

1. If starting from frozen stocks, partially defrost a vial of cells by warming in hand, and then add 1 mL pre-warmed RBL medium to fully defrost.
2. Transfer cells to a 25 cm² flask containing 8 mL RBL medium, and place in a cell culture incubator overnight.
3. When the cells are confluent in the 25 cm² flask, transfer cells to a 75 cm² flask by detaching adherent cells using trypsin–EDTA (*see steps 1–7 of Subheading 3.3*).
4. Maintain cells in 10 mL RBL medium supplemented with 1 mg/mL G418 sulfate and 20 µg/mL blasticidin S (NEAT-DsRed), in a 75 cm² flask, in the cell culture incubator.
5. Passage cells when confluent using trypsin–EDTA (*see steps 1–8*).
6. Freeze stocks of cells in larger batches. Centrifuge cells at $300 \times g$ for 5 min, and resuspend in RBL medium with 10% DMSO to obtain a cell concentration of 5×10^6 cells/mL. Aliquot 1 mL cells/cryovial, transferring cryovials to a Mr. Frosty[™] (Nalgene) container filled with isopropanol, and place in –40 °C for 1 h and then to –80 °C overnight. After freezing, transfer the cryovials to a liquid nitrogen Dewar flask for long-term storage. Defrost a vial to check the viability of the batch.

3.2 Treatment of Serum Before Sensitization

1. Heat aliquot of human serum at 56 °C for 5 min.

3.3 Sensitization

Cells are sensitized for 16–20 h by incubating them in RBL medium with the desired dilution of previously heat-inactivated serum.

1. Pre-warm RBL medium to 37 °C in a water bath.
2. Remove medium from 75 cm² flask of confluent cells.

3. Wash the flask once with 10 mL DPBS without Ca^{2+} or Mg^{2+} .
4. Aspirate and discard DPBS.
5. Add 2 mL trypsin–EDTA to the flask and tilt the flask from one side to another to ensure that the entire surface has been covered with the solution.
6. Incubate the flask for approximately 15 min at 37 °C in a cell culture incubator, until clumps of cells are visibly detached.
7. Add 8 mL pre-warmed RBL medium to the flask and gently resuspend the cells.
8. Transfer the cells in a sterile 50 mL tube.
9. Count the cells using a hemocytometer and calculate the total cell number.
10. Pellet the cells by centrifuging at $300 \times g$ for 5 min.
11. Resuspend the cells to a concentration of 3×10^5 cells/mL using RBL medium.
12. Sensitize cells using appropriate concentration of serum (*see Note 1*).
13. Plate 3 mL cells/well of a 6-well plate.
14. Incubate the plate overnight in the cell culture incubator.

3.4 Protein Blocking

1. Sterilize allergen printed surfaces using ultraviolet light in a Class II biosafety cabinet at 30,000 J/cm² for 30 min (*see Note 2*).
2. Carefully adjust the slides to the 16-well multi-well chambers (*see Note 3*).
3. Wash the surfaces 3× with 100 µL 0.05% v/v Tween 20 in DPBS (*see Note 4*).
4. Add 100 µL (1 mg/mL) BSA diluted in DPBS in each well. Incubate at 37 °C for 1 h.
5. Wash 3× with 100 µL 0.05% v/v Tween 20 in DPBS.
6. Wash 1× with sterile DPBS.
7. Add 100 µL (10 µg) fibronectin, diluted in DPBS, in each well of the multi-well chamber. Incubate at 37 °C for 3 h.
8. Wash 3× with sterile DPBS.

3.5 Stimulation

1. Collect sensitized cells using a sterile cell scraper (30 mm) (*see Note 5*).
2. Transfer 200 µL cell suspension in each well of the 16-well multi-well chambers (*see Note 6*).
3. Incubate for 24 h in a 37 °C/5% CO₂ humidified cell incubator.

3.6 Measurement of Activation

1. Gently remove the slides from each multi-well chamber (*see Note 7*).
2. Scan the slides using a GenePix® 4000B Microarray Scanner. Excitation laser light was set at 532 nm = standard green (PMT gain: 700), matching pre-installed emission filter standard green (558 nm to 593 nm; 575DF35575).
3. Using the GenePix Pro 6.1 software, circles can be drawn around every spot of interest and then automatically adjusted to size for every spot using the align features in the selected block mode function of the software. This allows the software to calculate the spot-specific fluorescence intensity. Care should be taken that the features are aligned correctly in order for accurate results to be obtained.
4. Once acquisition is completed, the image is interrogated using the GenePix Pro 6.1 software (Molecular Devices, US). Fluorescence intensity from every spot is then determined (Fig. 1).

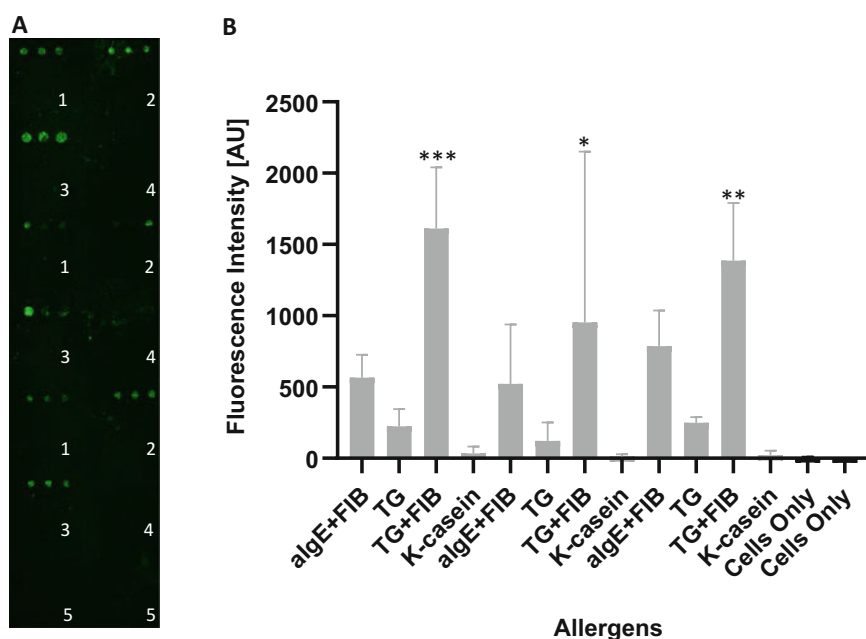


Fig. 1 (a) Cell binding of RBL 703/21 NFAT-DsRed to immobilized proteins printed in triplicate (1. anti-human IgE+10% fibronectin, 2. timothy grass (TG) pollen extract, 3. TG + 10% fibronectin, 4. k-casein, and 5. cells only) on amine-coated slides. (b) Fluorescence intensity values of individually activated protein spots on amine-coated slides. Data analysis was performed using one-way ANOVA (Dunnett's multiple-comparison test, $n = 3$, $* = p < 0.05$) compared to the negative control (cells only). RBL cells were sensitized with the serum of a grass pollen allergic patient overnight and incubated with the slides (8.4×10^5 cells/slide) for 24 h. Images of each protein spot were scanned using a GenePix 4000B Microarray Scanner at 532 nm laser wavelength

3.7 Data Analysis

1. Data is exported from the scanner using the GenePix Pro 6.1 software as a .txt file. Data analysis can then be performed using e.g. Microsoft Excel.
2. The fluorescence intensity of each allergen spot is determined via the subtraction of B_{532} values from the F_{532} value determined using the GenePix Pro 6.1 software. Each measurement was performed in triplicate (*see* **Note 8**).
3. Perform statistical analysis using GraphPad Prism 8 or comparable software.

4 Notes

1. Human sera are frequently cytotoxic for RBL cells. The presence of activated complement can be partially responsible, which can be traditionally inactivated by heating at 56 °C for 30 min. However, this prolonged treatment can result in irreversible conformational changes in IgE molecules, affecting its ability to bind to FcεRI. Thus, a shorter heat inactivation of just 5 min will be sufficient to inactivate complement while preserving the integrity of IgE. In our hands, even 20% serum concentration (1:5 dilution) has proven adequate to produce good results. In most cases, a 1:50 or a 1:100 dilution will be sufficient to remove cytotoxicity without any heat treatment. Beware, however, that increased serum dilution may reduce the sensitivity of the assay below the threshold needed for activation.
2. We advise sterilizing the slides, as well as the 16-well multi-well chambers, in a Class II biosafety cabinet in sterile petri dishes. After sterilization, securely close the petri dishes and seal with Parafilm. These can be kept at room temperature until needed and should be opened only while working in a sterile safety cabinet.
3. Carefully adjust the amine-coated slides onto the sterilized multi-well chambers. Do not apply too much pressure, as this might result in breaking the slides.
4. All washing and blocking steps are performed in a Class II biosafety cabinet. Open the lid of the sterile petri dish again in the safety cabinet. In new sterile petri dishes, pour the washing and blocking solution. Aspirate 100 μL using a multi-channel pipette and slowly release the content with the tips of the pipette touching only the walls of the multi-well chamber. Aspirate the content by touching with the tips the bottom edges of every well to repeat the washes.
5. Gently scrape the cells from the six-well plate without using too much force and/or pressure. To make sure the cells have

detached from the surface, use a 1 mL pipette and gently resuspend them in pre-warmed EMEM cell medium.

6. Add the 200 μ L cell suspension gently and slowly into the wells of the multi-well chamber. Excessive force might damage the cells and reduce cell viability.
7. Care should be taken when removing slides from the multi-well chambers as there is a reasonable chance that the slide may break. Try to remove one clip of the multi-well chamber at a time, gently applying some force with a sharp material at the edges. Allow air to move within the edges before lifting it up. Always wear nitrile gloves during this step.
8. In F_{532} Mean— B_{532} , the F and B parameters stand for feature pixel intensity and background intensity, respectively, where the feature is the printed spot and the background is the area immediately around the printed spot.

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