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Allergen-Based Diagnostic: Novel and Old Methodologies with New Approaches

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Abstract

This chapter is an extensive review of allergen-based diagnostic methodologies including old techniques such as skin prick test, radio-allergo sorbent test, enzyme-linked immunosorbent assay, and fluorescent-enzyme immunosorbent assay. Novel technologies include functional tests by flow cytometry and molecular allergy based on multiplex immunoassays. We also review the importance of biochemical characteristics of allergens, sensitivity and specificity, cross-reaction between allergens, utility, reproducibility, interpretation, and methodologies for discovery of epitopes for diagnostic or therapeutic use.

Keywords: allergens, skin prick test, RAST, ELISA, FEIA, ISAC, FACS, BAT, flow cytometry, antigen-antibody reaction, diagnostic

1. Introduction

Allergic diseases have been considered a worldwide health problem. The incidence and prevalence of hypersensitivity/allergy conditions are increasing every day, affecting people of any age, damaging a broad range of organs, and making a diagnostic challenge for the clinician [1]. It is well known that an accurate diagnosis could affect the clinical outcome; this is particularly important for the treatment of allergic diseases because the identification of the causative antigen and other molecules associated with specific immunological activation by allergen-based tests allows personalized medicine and precision treatment [2].

2. Historical perspective of allergen-based diagnostic methodologies

2.1. Skin prick test (SPT)

Skin prick testing is an essential clinical test to confirm sensitization in IgE-mediated allergic diseases. Historically, we can find an early report in 1850, in a textbook of Henry Salter, a physician from London's Charing Cross Hospital who described the formation of wheals following scratches in patients with asthma and exposed to cats [3]. In 1907, Clemens von Pirquet reported a modification of Koch's subcutaneous procedure based in abrasion of the skin to evaluate tuberculin response [4]. This procedure was rapidly adopted by others as a prototype for prick-puncture testing, and in 1909, the first case of anaphylactic response after scarification and exposition to an allergen was reported [5]. Practical application of a standardized procedure was suggested by Schloss [6] who described a correlation of time with clinical signs, reporting 5–15 min of erythematous reaction after abrasion of the skin in a child with rhinitis, asthma, and eczema. Since then, several techniques to evaluate allergenic sensitization have been described, e.g., intracutaneous test, [7] conjunctival test, [8] intracutaneous test by serial dilutions [9]. Nowadays the best technique to evaluate with safety allergenic sensitization is the SPT.

The standardized method of prick testing includes the appropriate selection of allergens, i.e., allergens tested are according to the country, the geographic location inside the same country, and even with seasons [10, 11]. SPT is based on the presence of sensitized cells, mainly mast cells in the skin, and the resulted cutaneous reactivity is used by the clinician as a surrogate biomarker for sensitization in eyes, nose, lung, gut, and skin. During the test, positive and negative controls must be included, a positive result is defined with a wheal ≥ 3 mm diameter after 15–20 min; reproducible results are obtained with standardized mixtures [12, 13]. In the early years of use, skin prick testing did not have with the entire approbation of the medical community, and their clinical relevance was questioned [14]. That vision has changed, and in the last years, it has been recognized a concordance between the clinical manifestations and allergen-specific wheal size [15]. Thus, skin prick test is considered as a fundamental technique to explore allergen sensitization in patients, but if it is true, why we need other methods to study sensitization in allergic/hypersensitivity conditions? In the following paragraphs, we will explain applications of the most common laboratory assays used to evaluate IgE specificity and the information obtained in functional allergen-based tests.

2.2. IgE and allergy

The discovery of the reaginic activity in the IgE antibody by Ishizaka in 1967 [16] developed a revolution in the knowledge of allergy affecting not only in basic research but also in applied research resulting in innovative diagnostic tools. It is well known that patients with allergy have a tendency to produce high levels of IgE antibodies due to its atopic condition. Usually, the concentration of total IgE in serum from healthy individuals ranges below 1 $\mu\text{g/mL}$. It is worthy of note that this is a very low concentration of protein so many laboratories rather use IU/mL or kU/L instead of $\mu\text{g/mL}$ to report IgE levels, but understanding that 1 kU/L equals

to 2.4 ng/mL [17]. Total IgE does not correlate with clinical manifestations, and is preferably to measure specific IgE (sIgE) [18, 19]. Total IgE concentration is the addition of all the specific IgE (sIgE) to the different allergens the individual has been exposed to; in non-allergic subjects, sIgE levels are below the limits of detection (0.35 kU/L) [20]. Thus, to identify the triggering antigen of allergic manifestations, one of the most common laboratory test requirement is the determination of sIgE concentration in serum. The quantification of sIgE can be performed through several methods based on antigen-antibody reaction, e.g., radio allergo sorbent test (RAST), enzyme-linked immunosorbent assay (ELISA) and fluorescence enzyme immune assay (FEIA).

3. Antigen-antibody reaction for IgE detection: general principle

Quantification of IgE is based on the antigen-antibody reaction, in which antigen is recognized specifically by an antibody forming an immune complex (**Figure 1**). In these tests, the antigen corresponds to serum IgE, and the antibody reacts against IgE in the serum sample. To carry out these assays, a specific antibody for the Fc fraction of the IgE is adsorbed into a solid phase usually in polystyrene or cellulose wells. This so-called “primary antibody” or “capture antibody” has the function of binding to the IgE in the sample of serum; this interaction generates a stable bound. Then, this immune complex is measured through a second antibody called “secondary antibody” or “detection antibody” which is coupled to a radioisotope (RAST) or an enzyme which allows the development of a colored (ELISA) or fluorescent (FEIA) substrate in an antigen-dependent manner. Simultaneously, a calibration curve containing known concentrations of the analyte to determine is processed to extrapolate the data of absorbance (in colorimetric methods—ELISA) or the fluorescence (in fluorometric methods—FEIA) to a protein concentration, finally reported in µg, ng, or IU.

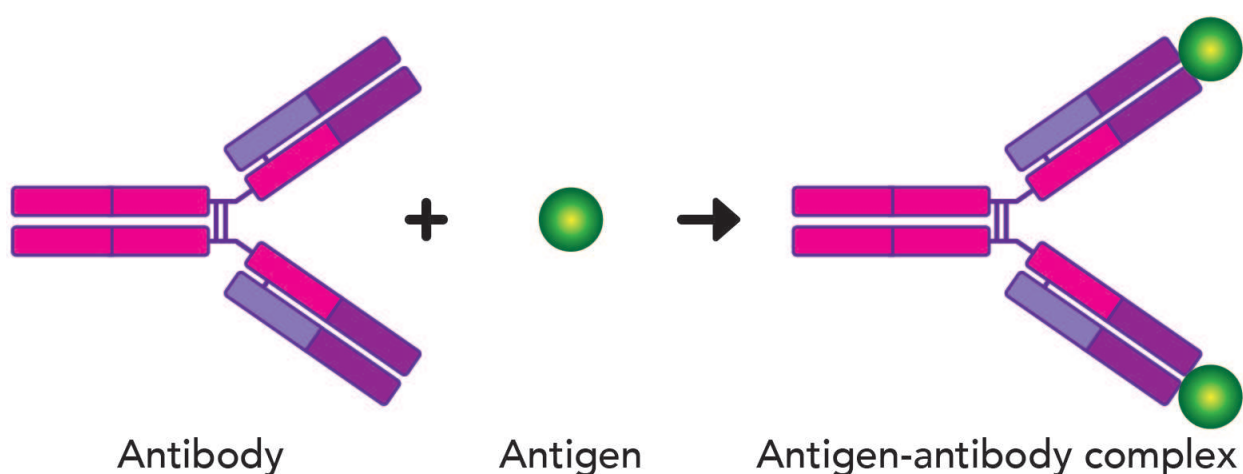


Figure 1. Antigen-antibody reaction. Each antibody is able to bind its specific antigen, forming antigen-antibody complexes. Different laboratory testing techniques are based on this principle.

3.1. Radio allergo sorbent test (RAST)

RAST was the first laboratory method developed for *in vitro* detection of specific IgE [21]; despite that it is no longer used and its historical importance is evident since it was the second most used test after SPT, and gave rise to development of new methodology to facilitate sIgE detection. The main advantage of this method over SPT lied on the safety of patient. In SPT, the allergen is administered in the cutaneous layer of the skin, which may lead to sensitization to new allergens or in the worst of cases it may trigger anaphylaxis. All of the previous disadvantages were avoided with the introduction of *in vitro* tests like RAST.

As mentioned above, this test is based on the principle of antigen-antibody reaction. In this method, the allergen is adsorbed covalently to a solid particle, then, the serum of a patient is added. IgE antibodies present in the sample binds to the adsorbed allergen. After this, a washing step is needed to remove non-specific weak bindings. Next, a radio-iodinated anti-IgE antibody is added to this reaction, and finally, the radiation detected is directly proportional to the number of antigen-antibody complexes formed (Figure 2).

This method was validated in comparison with sIgE in pollen-sensitized individuals, finding 96% of concordance with both tests [22].

3.2. Enzyme-linked immunosorbent assay (ELISA)

ELISA is currently one of the most common immune-assays used in clinical and experimental procedures. This technique allows detection of allergy-related analytes, e.g., IgE or Th2 cytokines, and screening of different molecules. Advantages of ELISA are fast performance, improved biosafety when compared with radioimmunoassay, low reagent cost, affordability for the patient, and simple methodology [23].

The first step to perform an ELISA is sensitizing the plate. A solid polystyrene plate is coated with an anti-IgE antibody directed against Fc region of the immunoglobulin (capture antibody). This process is achieved pre-treating the plate wells with carbonate buffers or cyanogen bromide allowing a better chance for adsorbing the capture antibody or antigen. Another

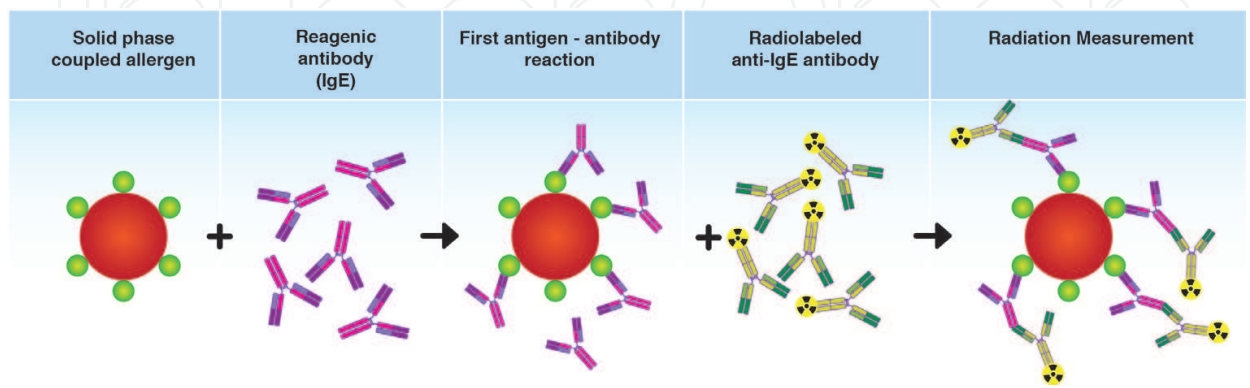


Figure 2. RAST methodology. An allergen is adsorbed covalently to a solid particle, then serum of patient is added to react with the allergen. Next, a radiolabeled anti-IgE antibody identifies the previous formed immune complexes. The radiation generated is measured by a radiation detector.

strategy is to radiate the polystyrene plate; this permits the breaking of a certain number of benzene rings yielding carboxyl (COOH) and hydroxyl (OH) groups. Radiation of polystyrene increases the chances for hydrophilic interactions with Fc fractions of capture antibody. Protein A from *Staphylococcus aureus* is also a suitable linker for orienting and spacing the capture antibody appropriately, optimizing the space, and homogenizing the coating. In this step, several factors like pH and temperature could affect the proper adsorption of antibody or allergen. Fortunately, commercial kits contain the pre-sensitized plates. Next step is incubation of samples (serum or plasma), although serum samples are preferred over plasma samples since some commercial houses have documented diminishing IgE detection sensitivity in plasma samples.

Incubation let the captured antibody bind to IgE through Fc fraction (in total IgE determination) or allows the specific IgE contained in the sample bind to the allergen adsorbed in the solid phase (in specific IgE determination). After incubation time, a washing step is performed to remove weak and unspecific binding. Then, a second anti-Fc antibody is added to the well to detect the immune complexes formed in the previous step. This “secondary or detection antibody” is linked to an oxidative enzyme that acts on its substrate which once oxidized develops a color that can be measured through a spectrophotometer (**Figure 3**).

There are various enzymes and substrates commonly used in ELISA (**Table 1**); the biotin-streptavidin system is the most often employed in detection methods. A washing step is followed next to eliminate the excess of not bound antibodies. In addition to samples, control or standard curve is processed with increasing concentrations of protein. The goal of this standard curve is to extrapolate the absorbance obtained from samples into a curve of known concentration through a linear regression, and obtaining an estimated concentration of the analyte (**Figure 4**).

The last step consists in adding and incubating the substrate for 10–20 min and reading the absorbance obtained after stopping the reaction. Some substrates may be read without stopping

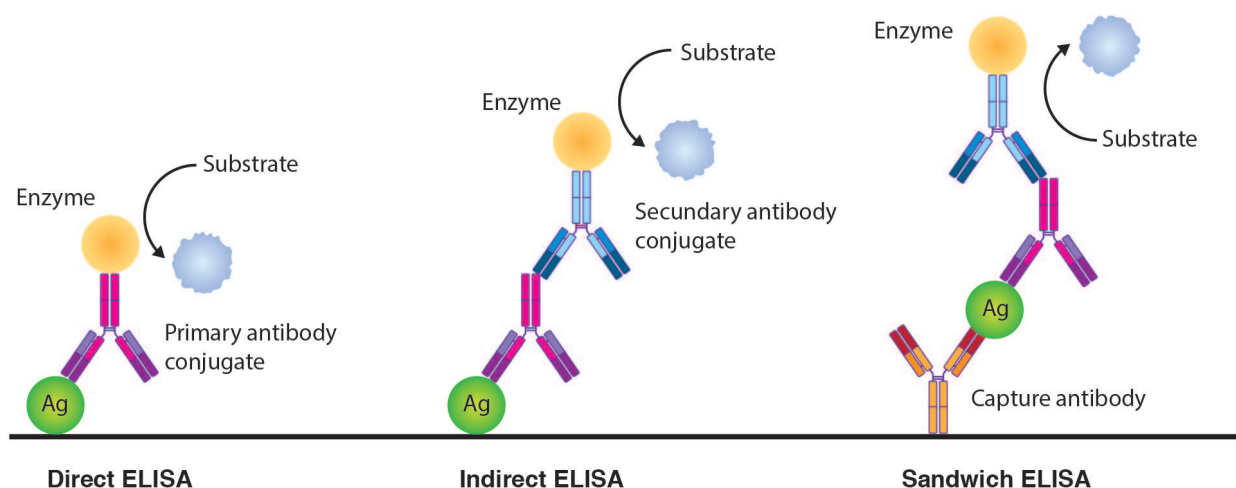


Figure 3. ELISA assays. Direct ELISA mostly used for antigen detection. Indirect ELISA mainly used for antibodies detection. Sandwich ELISA used to detect total IgE.

Enzyme	Substrate	Wavelength after stop solution (nm)
Horse radish peroxidase (HRP)	3,3',5,5'-tetramethylbenzidine, TMB	450
	2,2'-Azinobis (3-Ethylbenzothiazoline-6-sulfonic acid) diamonium salt, ABTS	450
	o-phenylenediamine dyhidoclride, OPD	490
Alkaline phosphatase (AP)	p-Nitrophenyl phosphate disodium salt	450

Table 1. Enzymes and substrates used in ELISA assays.

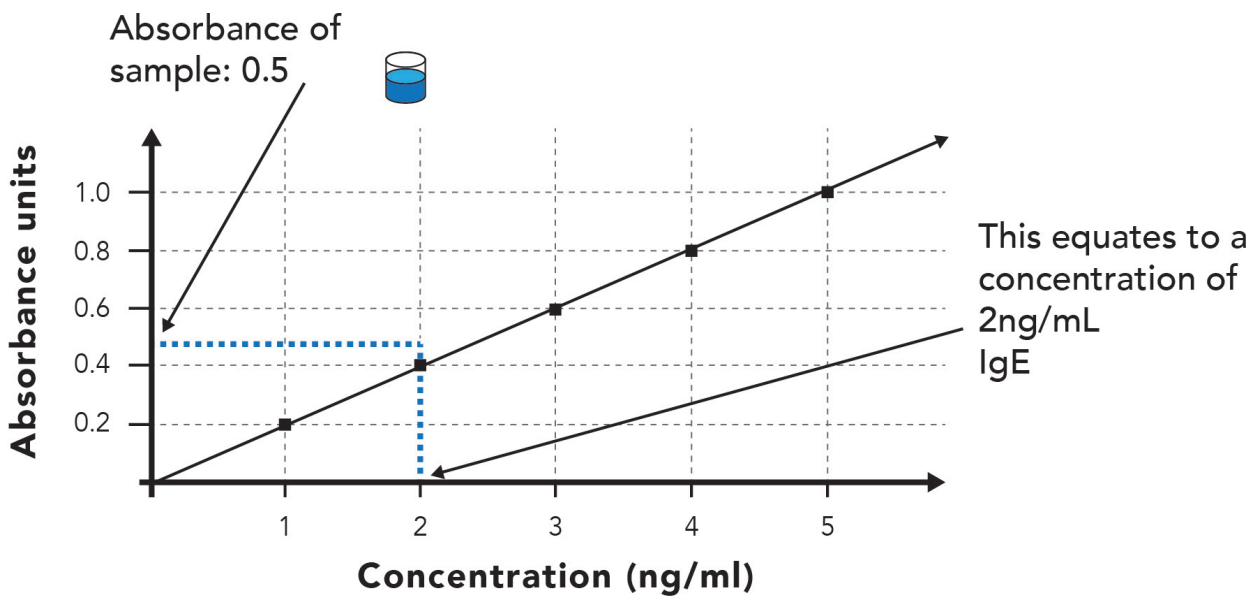


Figure 4. Absorbance-concentration standard curve. In order to determine sIgE concentration in a sample, a standard curve is run using known concentrations of total or allergen-specific IgE. By plotting the absorbance from patient samples into the standard curve, we can determine sIgE concentration.

the reaction. Results are read in a spectrophotometer; this equipment works emitting a light beam that is filtered through a wavelength selector or filter; then, the filtered light will strike the sample, which will absorb a certain amount of light and let some light pass and reach the detector. Absorbance is the negative logarithm of transmittance, so the absorbance obtained will be proportional to the concentration of the measured antigen-antibody complexes in the samples that may reflect and refract the light at a certain wavelength (Figure 5). Optical density (OD) is a common term used to refer to absorbance (see Figures 4 and 5).

ELISA rapidly substituted radiolabeled methods, due to its safer and faster performance, with a similar sensibility and specificity when compared to RAST and paper radio immuno-sorbent test (PRIST) for total or specific IgE quantification [24, 25].

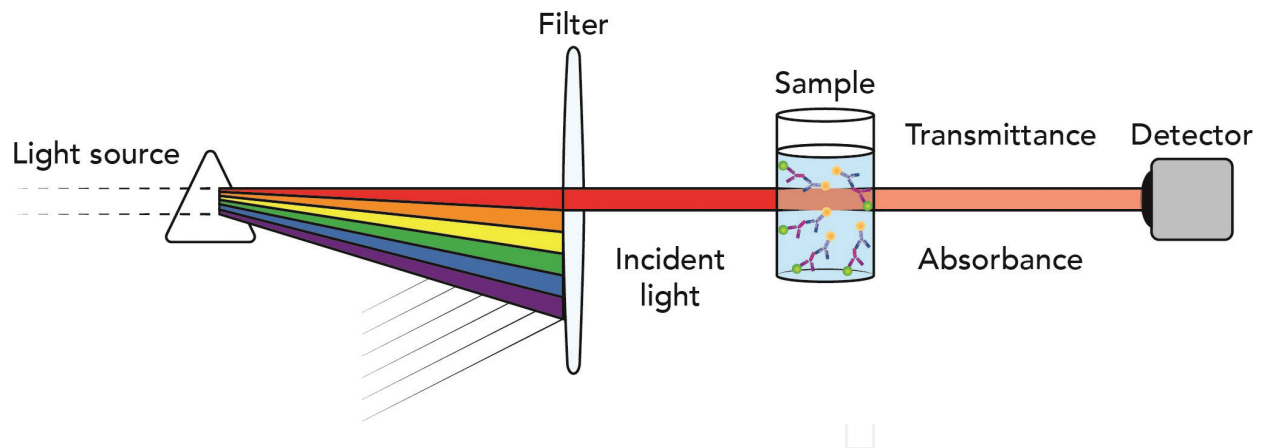


Figure 5. Spectrophotometer basis. A light beam is generated by a halogen lamp, and this light is filtered through a wavelength selector. Light hits the sample and the transmittance measured is reported as absorbance units. Absorbance is directly proportional to the amount of antibody-antigen complexes formed previously.

3.3. Fluorescent enzyme immune assay (FEIA)

Fluorescent enzyme immune assays are based on the same principle used for ELISA and RAST, the antigen-antibody reaction, but differs in the way the read out is made. In FEIA, the secondary antibody is linked to an enzyme that permits the activation of a fluorochrome. The most common enzyme used in fluorometric assays is β -galactosidase, which acts on its substrate 4-methylumbelliferyl- β -D-galactoside transforming it into a 4-methylumbelliferone. When 4-methylumbelliferone is excited at 365 nm, it emits fluorescence at 445 nm. This fluorescence is later measured by a fluorometer [26]. Simultaneously, a standard curve is processed to extrapolate the relative fluorescence units obtained from samples into the known concentration curve (**Figure 6**).

Fluorometric assays have permitted the development of automatized systems, resulting in improved reproducibility, diminished operator involvement, with reduction of mistakes, and increased sensitivity and specificity when compared with other innovative methods based on chemiluminescence [27, 28]. FEIA technology opens the possibility to screen sIgE to several allergens at the same time and with few volume of sample [29]. **Table 2** shows a comparison between antigen-antibody reaction-based methods for quantification of sIgE.

3.4. ImmunoCAP-ISAC (immuno solid-phase allergen chip)

Innovative and non-invasive techniques led to the identification of many sIgE to different allergens at the same time, with a minimum sample volume ($\sim 50 \mu\text{L}$), allowing test allergens not limited to a geographical region, and without risk of sensitization, or anaphylaxis for the patient, as has been reported for SPT [30].

ISAC is the first multiplex diagnostic tool commercially available to evaluate sIgE directed against 112 well-characterized antigens. In fact, the allergens presented in the solid phase are recombinant proteins ensuring specific interaction of serum IgE with higher accuracy when

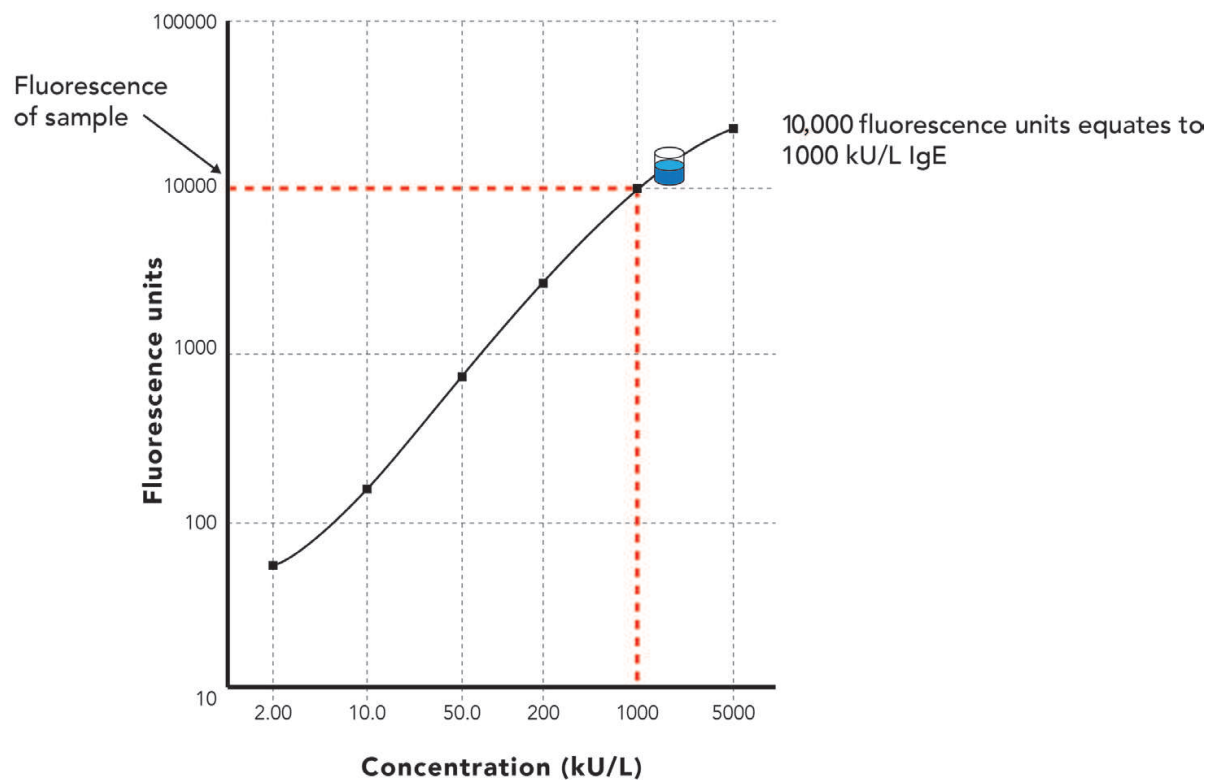


Figure 6. Fluorescence-concentration standard curve. Quantification of sIgE is measured plotting fluorescence units obtained from samples into a standard curve.

Test	RAST	ELISA	FEIA
Specificity	Depends on antigen preparation	High	High
Sensitivity	High	High	High
Reproducibility	Acceptable	Acceptable	High
Automatization	Partially	Partially	Totally
Relative cost per test	Low	Affordable	High
Shelf life of reagents	Long	Low	Long
Health hazards for laboratory personnel	High	Non or minor	Non or minor

Table 2. Methods for sIgE quantification based on the antigen-antibody reaction.

compared with FEIA and SPT [31]. The assay consists of various steps. First, the sIgE from serum samples interacts with the recombinant allergen previously adsorbed to the solid-phase; then, a secondary anti-human IgE antibody labeled with fluorochrome recognizes sIgE-recombinant allergen complex (**Figure 7**). Fluorescence is measured using a biochip; and results are scanned and analyzed in specialized software, reporting results in arbitrary units named ISAC Standardized Units (ISU) (**Figure 8**) [32].

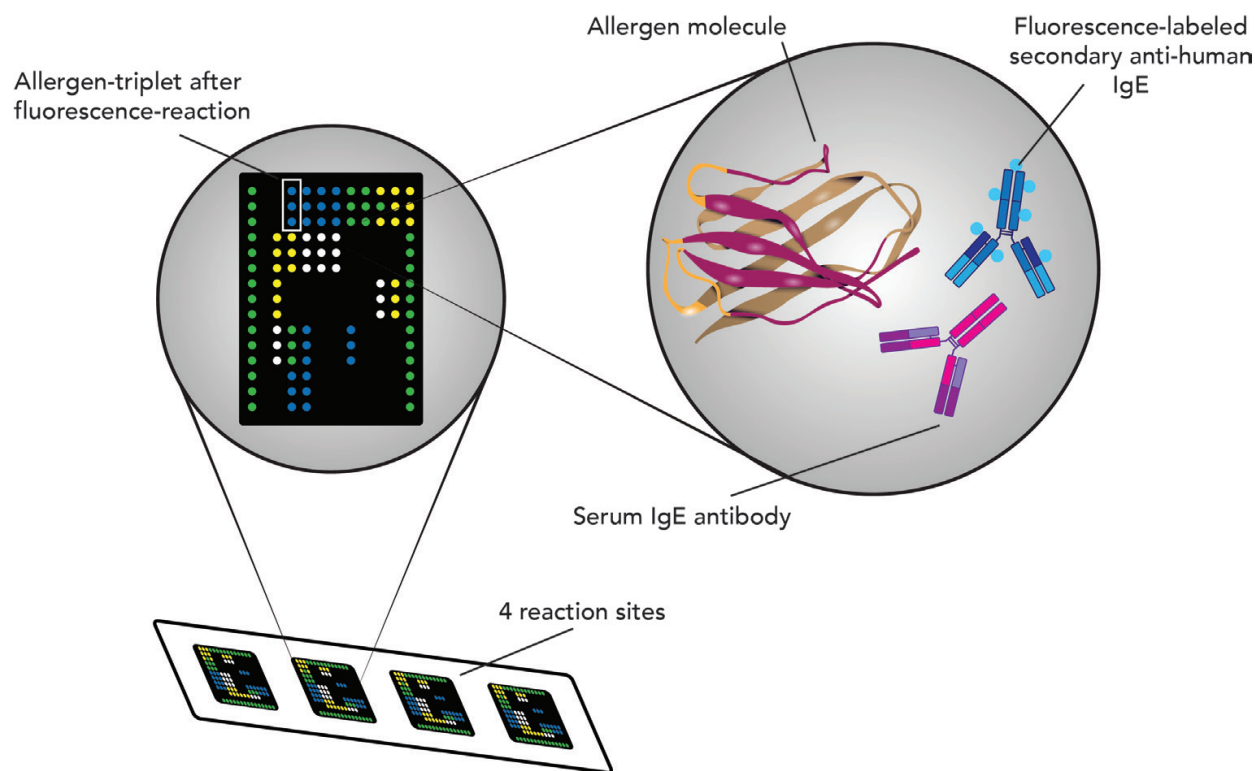


Figure 7. Immuno CAP-ISAC. The recombinant allergens are recognized by sIgE from serum samples; a secondary antibody fluorescent-labeled interacts with IgE. Fluorescence is measured by a biochip and results are analyzed in specialized software. Recombinant allergen diminishes the risk of cross-reactivity.

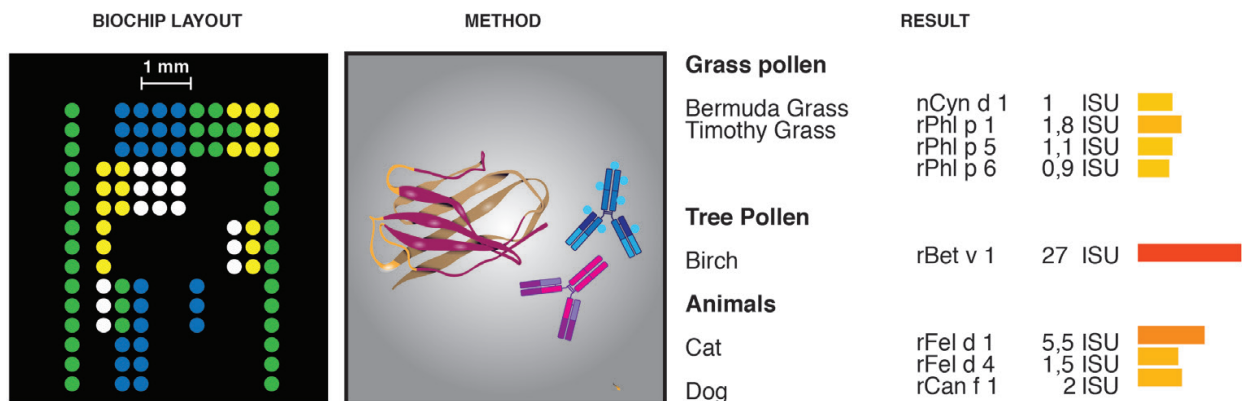


Figure 8. ISAC biochip layout. Results are reported in arbitrary units named ISAC-Standardized Units (ISU).

ISAC multiplex assay has been proposed to guide therapeutic decisions, e.g., the discontinuation of restrictive diets, the content of allergen-specific desensitization immunotherapy that may be useful to discriminate structurally similar allergens and cross-reactivity, and even to analyze the real sensitization profile in multi-sensitized patients to define whether they can receive a specific immunotherapy [33].

3.5. Western blot

Western blot combines different techniques to identify new antigens related to allergy. In this method, the antigens are separated according to their molecular weight in a sodium dodecyl sulfate polyacrylamide gel (SDS-PAGE), and then transferred to a polyvinylidene difluoride or nitrocellulose membrane, which will function as the solid phase for the antigen-antibody reaction. Then, the membrane is incubated with the patient serum, if sIgE is present in the sample it will react against the allergens found. A secondary anti-IgE antibody coupled to an enzyme is added (**Table 1**). Detection of sIgE becomes evident by the formation of bands in two different ways:

- (a) Developing color. The enzyme oxidizes the substrate and precipitates (e.g., when using a secondary antibody conjugated to horse radish peroxidase (HRP) and 4-cloronaphthol).
- (b) Releasing light. The substrate is dephosphorylated by an enzyme, releasing light (chemiluminescence), that is later detected by a photographic film or autoradiography (i.e., when using a secondary antibody conjugated to alkaline phosphatase (AP) and adaman-tyl-1,2-dioxetane phosphate or HRP and luminol) (**Figure 9**). Finally, concentration can be estimated by densitometer analysis.

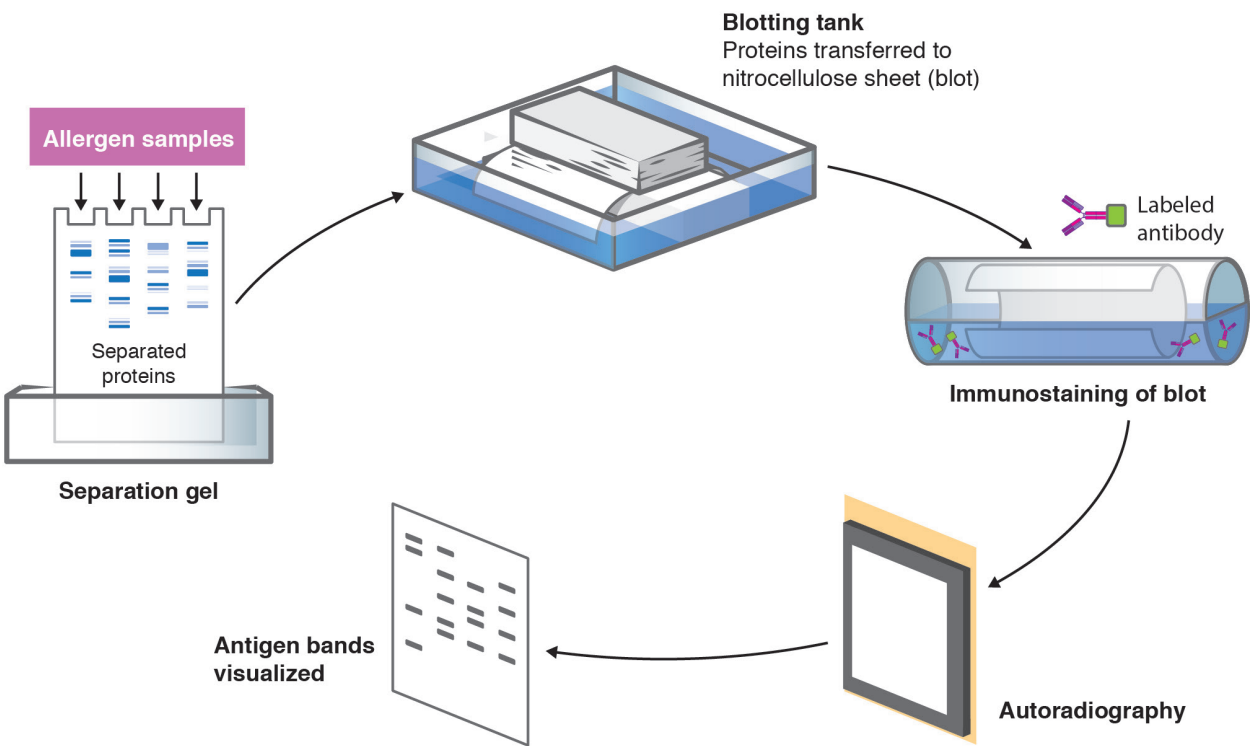


Figure 9. Western-blot methodology. Allergen mixtures are separated in a SDS-PAGE according to the molecular size. The separated allergens are transferred to a nitrocellulose or PVDF membrane. Then, by adding the antibodies from the serum samples sIgE will bind to their specific antigen. An enzyme conjugate secondary antibody identifies Fcε IgE. Detection of reaginic antibodies is identified by chemiluminescence.

This method has been useful in the identification of clinically relevant immunogenic epitopes after enzymatic digestion of allergens and is also used to identify cross-reacting peptides [34–36].

4. Limitations of sIgE determinations: allergen cross-reactivity

Something to consider when handling sIgE quantification is the cross-reactivity of certain allergens. Some allergens share amino acidic sequences that can be recognized by the same IgE antibody, and this phenomenon is called “cross-reactivity.” Cross-reactivity occurs mainly in aeroallergens and food allergens. It is considered to have a high chance of cross-reactivity when two allergens share 35% homology in an 80-amino acid sequence or full identity in a 6–8 amino acid peptide; also, there may be a cross-reaction when the IgE is specific for carbohydrate moiety in the allergen. Hence, laboratory blood tests may detect antibodies to allergens even if the patient has never been exposed to them [37].

Importantly cross-reactivity can occur between allergens from the same family like in nut allergens or in different species of house dust mite; but also, cross-reactivity could be present in diverse phylogenetic sources like house dust mite and shrimp, birch and apple, or fish and chicken meat (**Table 3**) [38–40]. As we read in the previous section, technology innovation through recombinant allergens and full automatization notably reduce cross-reactivity risk when performing sIgE determinations.

Cross-reactivity	Antigens involved on cross-reactivity
Birch-apple	Bet v1 homologue Mal d1
Cypress-peach	Pru p3 non-specific lipid transfer protein (LTP)
Celery-mugwort-spice	Art v4 profilin, Art v60 kDa homologue to Api g5
Mugwort-peach	Art v4 profilin, Art v3 LTP
<i>Alternaria</i> -spinach	Alt a1
Mite-shrimp	Der p10, tropomyosin
Cat-pork	Fel d2 cat serum albumin
Bird-egg	Gal d5 alpha-livetin (chicken serum albumin)

Table 3. Allergen cross-reactivity and antigens involved.

5. Functional tests

The techniques above described answer two simple requests: is the sIgE present in the sample? So, if there, how much sIgE is present? The answer to these questions and the analysis of the clinical history allows the allergist/immunologist to initiate treatments centered on allergen-specific desensitization in every single patient in a personalized way. However, sometimes

answer to these questions is not enough, and functional tests are needed to understand some clinical manifestations, e.g. allergy to a particular drug.

5.1. Flow cytometry and fluorescence-activated cell sorting (FACS)

Early in the 1950s, Coulter developed a technology able to read size and complexity of blood cells based on diffraction of light laying the fundamentals for automatized blood counting used in our days. Exploiting this innovation, Bonner, Sweet, Hulett, Herzenberg invented the Fluorescence Activated Cell Sorter (FACS) in the late 1960s to achieve flow cytometry and cell sorting of viable cells. Becton Dickinson with Bernie Shoor introduced the commercial cytometers in the early 1970s, utilizing a Stanford patent and the expertise supplied by the Herzenberg Laboratory [41]. Today, isolation of cells by FACS is performed in complete sterility, and sorted cells could be used as an adoptive transfer for therapeutical interventions [42].

Flow cytometry detects and analyzes optical signals (angular light scatter or emitted fluorescence) to identify individual characteristics of cells or in biological samples. Inside the flow cytometer, the suspended cells are conducted in a fluidic system ensuring cells travel at a uniform velocity in a laminar form. Here, the cells are directed to a specific point in which a laser passes through cells. The light is diffracted in all directions, the emitted light is recovered in filters, and photodetectors collect the detection signals. The optical detection system obtains information about forward light scatter (FSC), side light scatter (SSC), and fluorescence channels (FL1, FL2, FL3). Then, the luminous signal is detected in photomultiplier tubes; information recollected is digitalized that is to be analyzed by a computer system. Information obtained is showed in histograms or dot plots. The quality of both systems (optical and fluidic) is critical for performance and reliability of this technique [43] (**Figure 10**).

Flow cytometry could be used to determine the expression of cell surface markers, to know absolute or relative numbers of cells, to determine intracellular proteins, to quantify soluble proteins, or combine all of these possibilities.

- (a) Expression of cell surface markers. Information obtained by analysis of expression of cell surface markers could be useful to know the cellular phenotype and some functions of labeled cells. A few examples include the state of activation of a particular cell [e.g., CD63 on basophils after drug exposition (see the next section of this chapter for a deeper explanation of basophils activation test)], to know absolute numbers of circulating cells (e.g., 1700 CD4/ μ L), or combining information (e.g., patients with ocular allergy have increasing percentage of circulating helper activated CD4+CD25+ T cells) [44].
- (b) Determination of intracellular proteins. This procedure is useful to assess specific functions of the cell. First, isolation of cells is needed prior incubation with a stimulus (e.g., allergens as specific stimulus). Culture or incubation conditions must be standardized to ensure reproducibility of results. It is important to note that if studied protein is secreted (e.g., cytokines) protein secretion must be inhibited (e.g., brefeldin-A that blocks internal protein transport) to allow retention of proteins inside the cytoplasm. Labeling of intracellular proteins is performed after cells were fixed and permeabilized with detergents (e.g.,

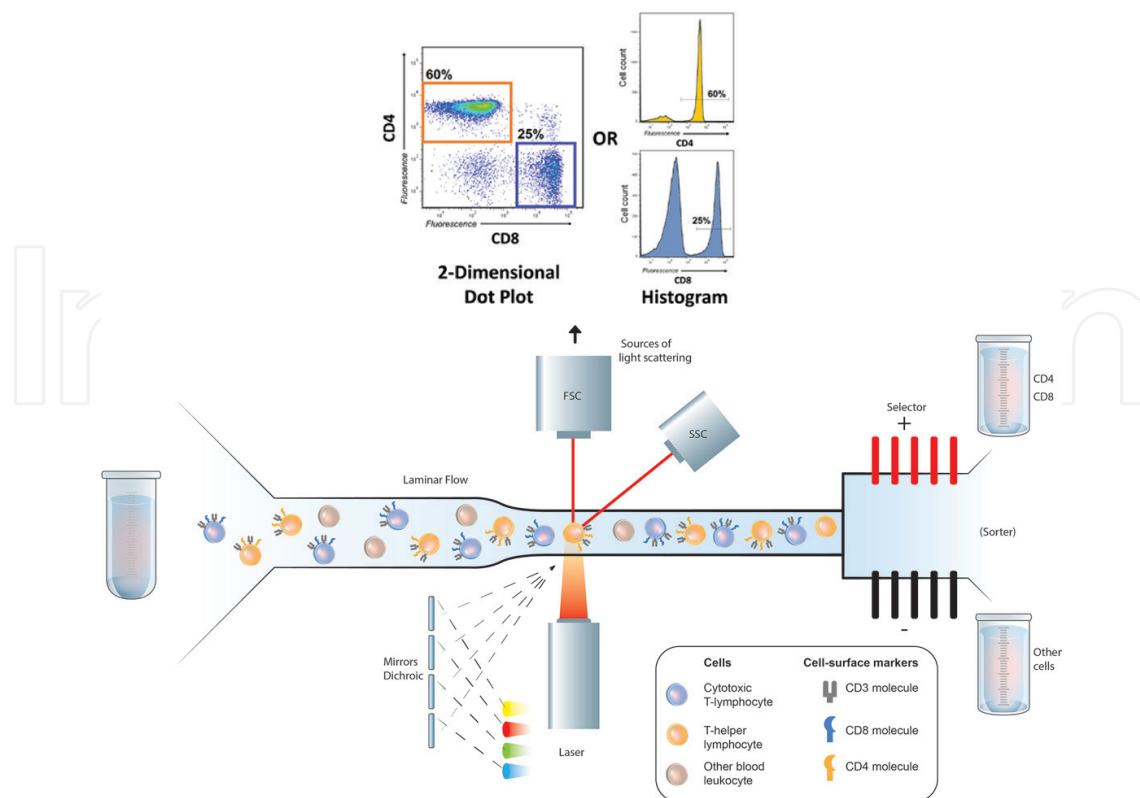


Figure 10. Flow cytometer and fluorescent activated cell sorter. The figure resumes the optical and the fluidic systems working together to analyze biophysical characteristics of cells, expression of molecules detected by monoclonal antibodies, and sorting of cells expressing selected characteristics.

saponin). Permeabilization process ensures that monoclonal antibodies (mAbs) labeled with fluorochromes enter into the cell and react with their specific antigens (**Figure 11**) [45]. The determination of intracellular proteins has significantly contributed to the understanding of physiopathology induced by allergens (e.g., Allergen-activation induces cytokines related to the damage of IL-25 in asthma, IL-31 in atopic dermatitis, and IL-5 in vernal conjunctivitis) [46–48].

- (c) Quantification of soluble proteins. The determination of soluble proteins could be used to know normal ranges of proteins in human fluids or to assess cellular functions. Multiplex technology has been developed to detect several proteins in the same sample, and it is named cytometric bead arrays (CBA). The advantage of this test is the low volume of sample letting to process a broad range of human fluids/secretions (e.g., tears, synovial fluid, aqueous humor, and serum) and cell supernatants [49–52].

Multiple determinations of soluble proteins by flow cytometry are based in microspheres, all of them conjugated with a specific antibody against protein we wish to determine. After bead interacts with its antigen, a second antibody labeled with a fluorochrome is added; usually, this secondary antibody is conjugated to phycoerythrine (PE). However, the real innovation of this assay is that each bead is also labeled with a different fluorochrome in a range of intensity, from low intensity to high intensity, and detected by near infrared (NIR) lasers [53, 54] (**Figure 12**).

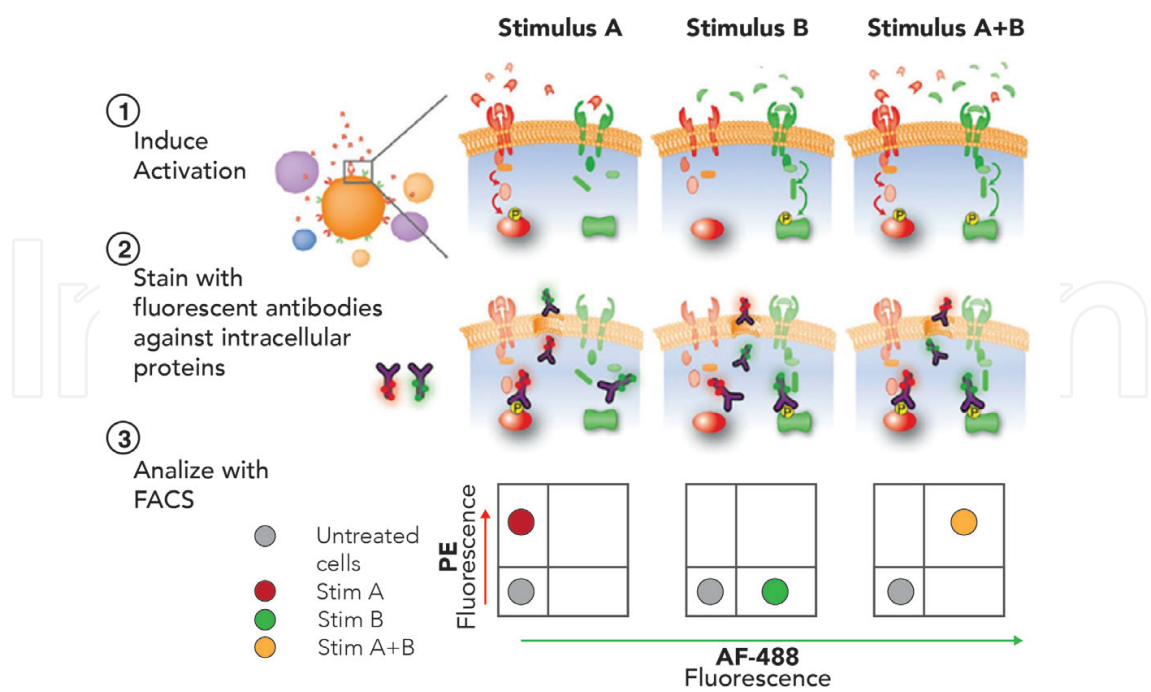


Figure 11. Determination of intracellular proteins by flow cytometry. Identification of intracellular proteins allows studying cellular functions.

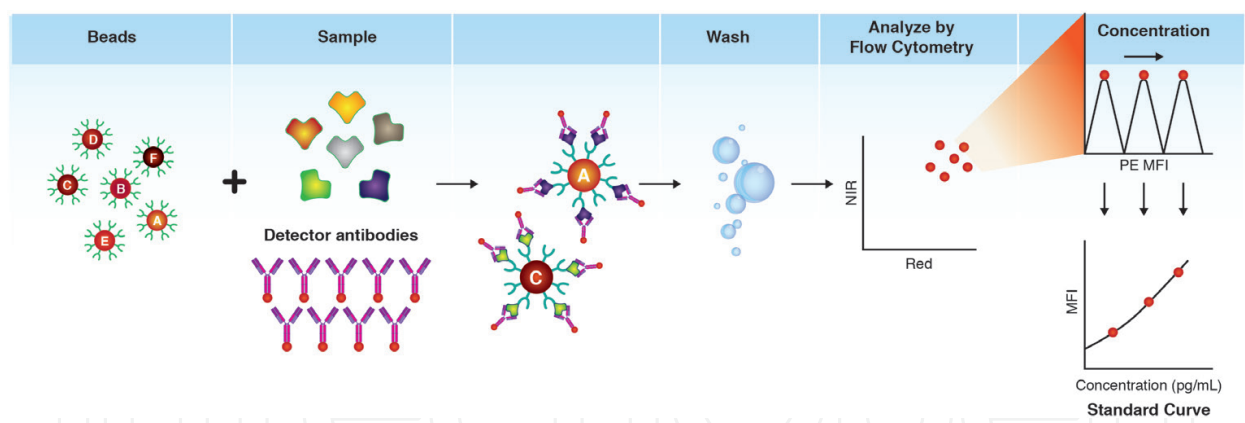


Figure 12. Cytometric bead arrays. Multiplex technology permits determination of various soluble proteins at the same time, and in the same sample.

Changes in intensity of fluorescence are expressed as median fluorescence intensity (MFI) and directly correlate with concentration of protein in the sample expressed in pg/mL or ng/mL (Figure 13).

5.1.1. Basophils activation test (BAT)

Adverse drug reactions (ADR) constitute a major health problem worldwide with high morbidity and mortality rates, the incidence of fatal ADR occurs in 5% in hospitalized patients in Europe [55]. ADR may be classified as Type A (augmentation of normal drug effects), Type B

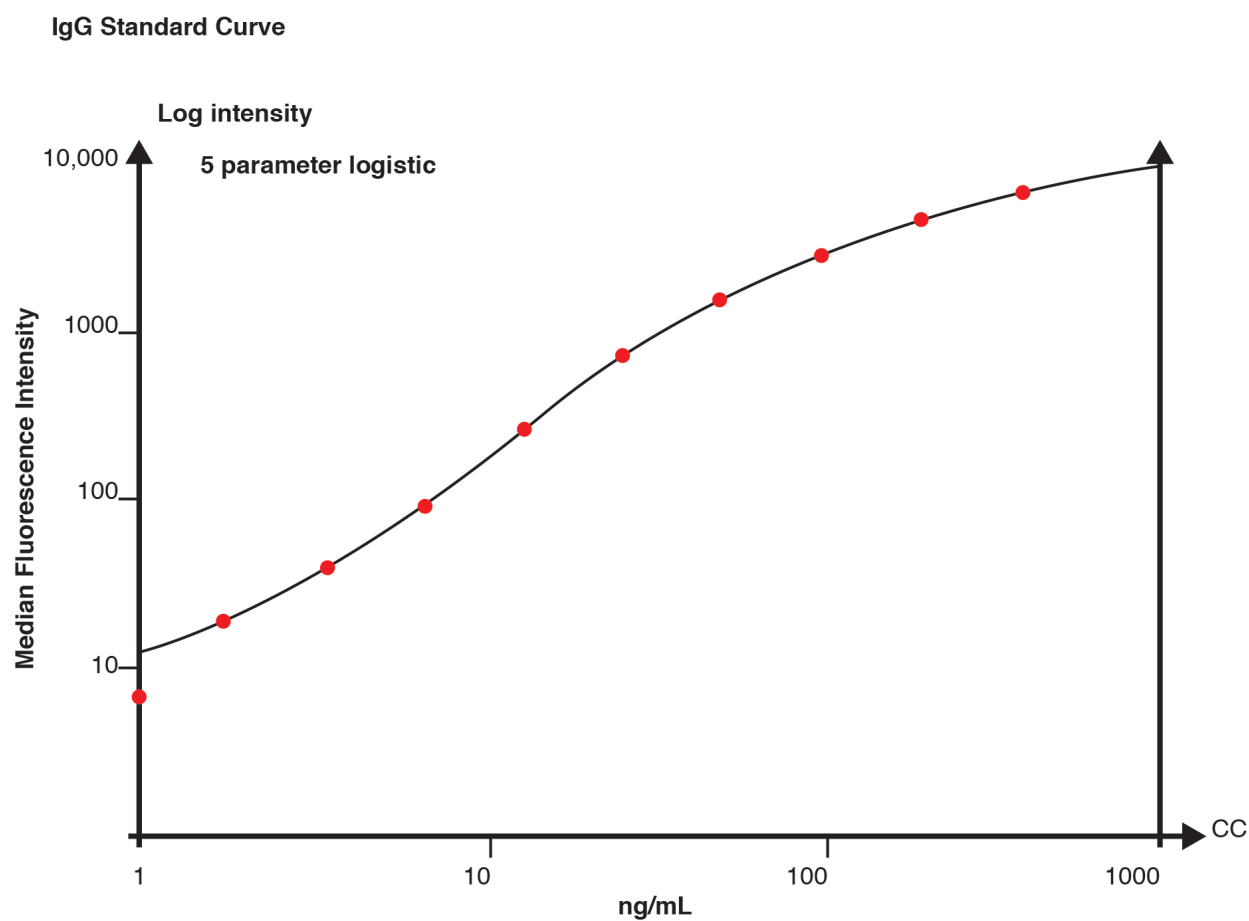


Figure 13. Standard-curve and median fluorescence intensity (MFI). Changes in MFI correlate with concentration of soluble protein.

(bizarre effects), Type C (chronic effects), Type D (delayed effects), and Type E (end of drug use effects). The most frequent ADR are Type A and are related to genetics, age, sex, and disease, and they have low mortality and high morbidity; in contrast, Type B are 25% of ADR and are unpredictable, with high mortality and low morbidity. The pathophysiological mechanisms of Type B reactions are not well understood. Some cases are mediated by type I hypersensitivity (true allergy), but other cases are related with the generation of reactive metabolites that react non-enzymatically on multiple proteins to form immunogenic-drugs complexes that induce a cascade of cell-based reactions and result in a wide range of severe clinical symptoms [56]. Due to the complexity of ADR, only Type B reactions could be explored by basophil activation test (BAT).

Principle of this test is simple, basophils are activated *in vitro* by the suspicious drug; if basophils are sensitized to the drug, they become active, upregulating on their surface two molecules CD63 and CD203c [57]. CD63 is an intracellular lysosomal protein whose surface expression is upregulated after activation. CD63 is also expressed on activated platelets, degranulated neutrophils, monocytes, macrophages, and endothelium [58]. On the other hand, CD203c is an ectoenzyme located both on the plasma membrane and in the cytoplasmic compartment of basophils. Cross-linking of the FcεRI by an allergen or anti-IgE antibody results in a rapid upregulation of intracellular CD203c molecules to the cell surface and is accompanied by mediator release [59] (**Figure 14**).

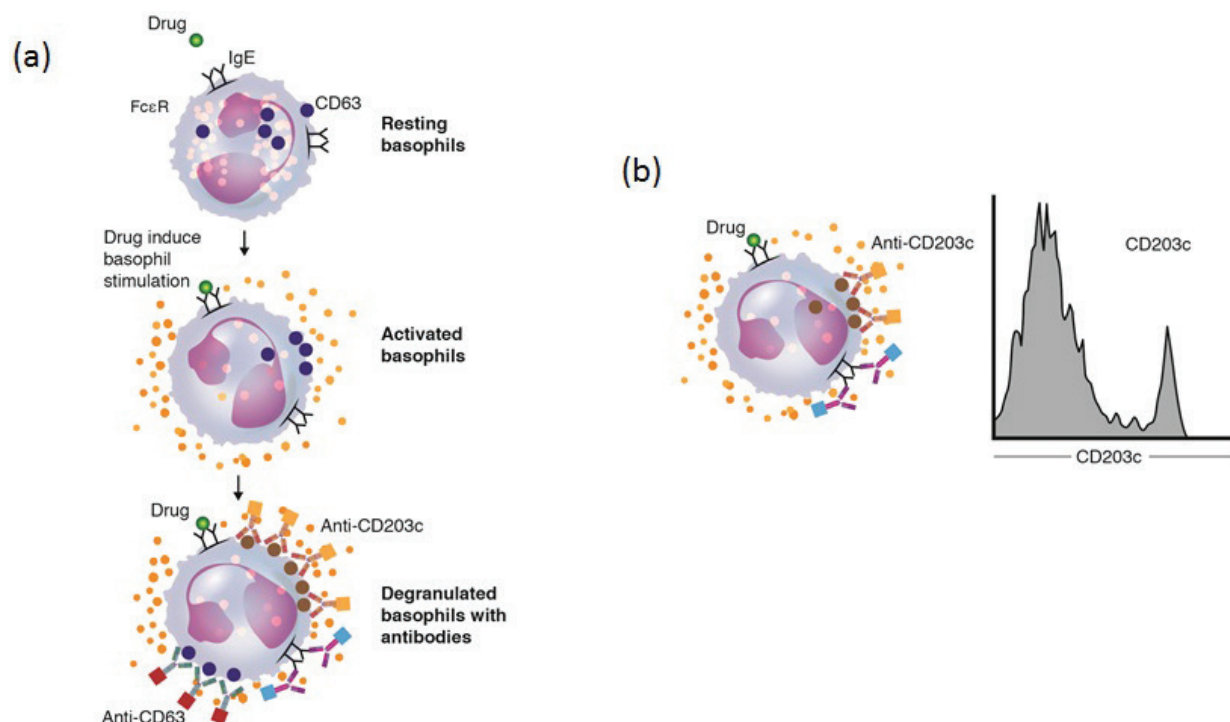


Figure 14. Basophils activation test. (a) After activation basophils upregulate CD63 and CD203c on membrane surface. Both CD63 and CD203c are detected by antibodies conjugated to fluorochromes; (b) the histogram shows increased expression of CD203c on gated basophils.

Reports about sensitivity and specificity for BAT indicate that determination of both, CD63 and CD203c, considerably increases the sensitivity up to 92% and specificity in a range of 86–90% [60, 61]. Today, BAT is also used to determine sensitization to several allergens such as diverse types of pollen and house dust mites. It has been reported that BAT has the same sensitivity but lower specificity when compared with FEIA. BAT could be used as an alternative to SPT in some patients with allergy to aeroallergens [62] and as a useful test preventing preoperative anaphylaxis [63].

BAT assay is performed with 100 μ L of peripheral blood; the drug is incubated with the blood at 1 mg/mL, in 36.5°C of temperature and atmosphere of 5% CO₂ during 1 h; as an internal control, the same volume of blood is incubated with negative or positive controls. N-formyl-methionyl-leucyl-phenylalanine (f-MLP) is used as positive control. f-MLP is an N-formylated tripeptide that functions as a chemotactic peptide for polymorphonuclear (PMN) cells but is a potent activator of basophils too. After incubation, cells are labeled with monoclonal antibodies for 30 min, and then erythrocytes are lysed and results are analyzed by flow cytometry. To ensure that CD63 expressing cells are basophils, analyzed cells are also labeled against CD123 and Human leukocyte antigen-DR (HLA-DR). CD123 is the IL-3R α , granulocytes including basophils, that constitutively express this cluster of differentiation [64]; whereas HLA-DR is expressed on B lymphocytes, monocytes, macrophages, activated T lymphocytes, activated natural killer (NK) lymphocytes but is absent in basophils. First, we analyzed cells by their complexity (SSC) and expression of CD123 and HLA-DR, basophils would be CD123+HLA-DR–, and only if activated by allergen or drug-medication, basophils would be CD63+ CD203c+ (SSC/CD123+HLA-DR–CD63+CD203c+) (**Figure 15**).

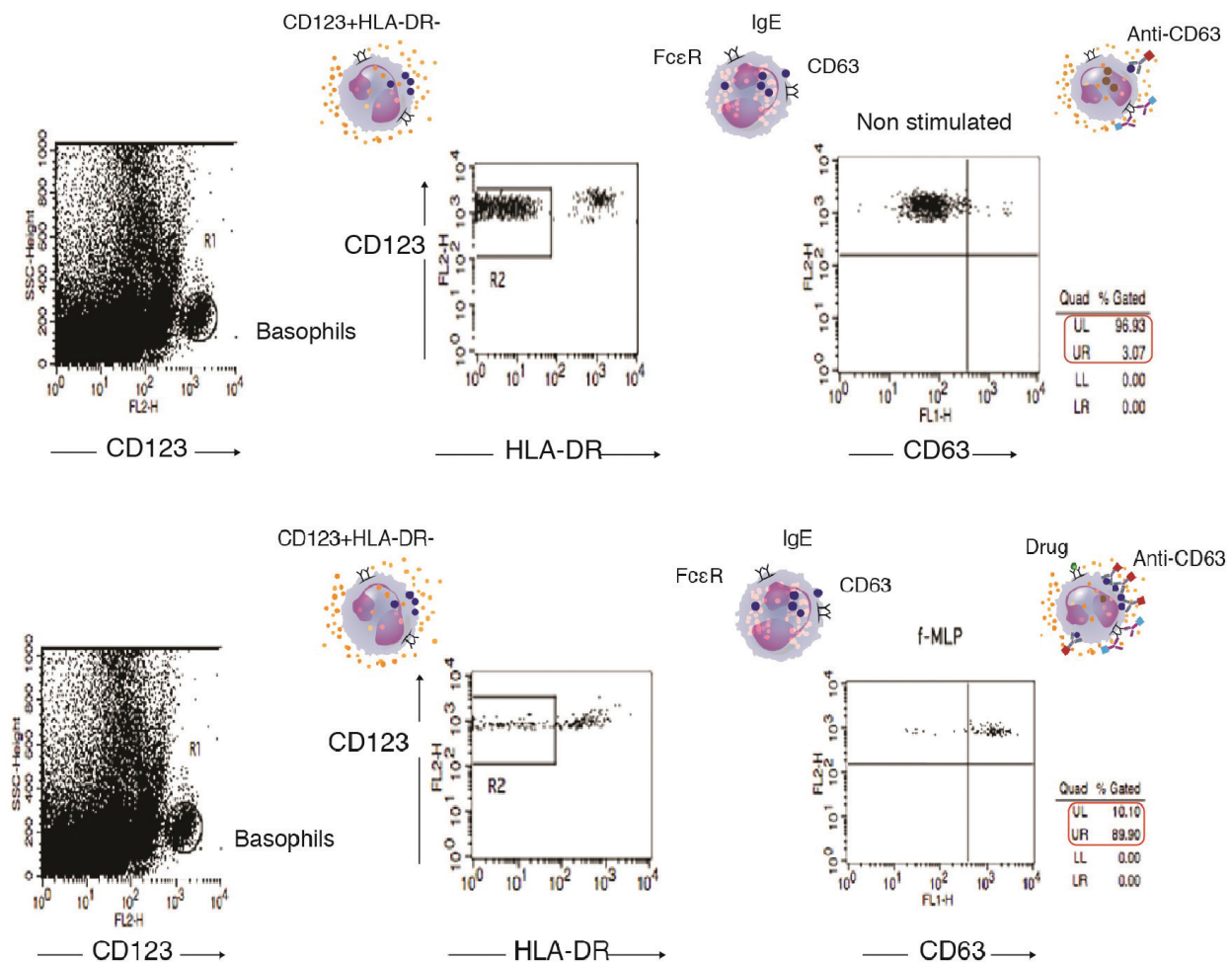


Figure 15. Representative dot plot of flow cytometry analysis of a basophils activated test. Upper panel shows non-stimulated cells. The lower panel shows stimulated cells with f-MLP. Percentage of activated basophils is showed at the squares next to dot plots.

6. Conclusions

The analytical and functional methods described in this chapter are evolved significantly since the first clinical report related to the identification of a triggering allergen in an asthmatic patient. All the allergen-based diagnostic methodologies revised in this chapter are grounded in the antigen-antibody reaction; recognizing the advantages and disadvantages of each analytic method is essential to make adequate choices. Although the apparent simplicity of methods is described here, some technical considerations have to be considered to avoid human errors when performing and interpreting sIgE tests.

It is important to note that the understanding of these techniques could be easy, but to apply them to make therapeutical decisions is not as easy. Allergic diseases are the best example of precision medicine. In this context, the therapeutical interventions through allergen-specific desensitization and addition of biologicals to block the function of certain molecules must be argued not only with evidence-based medicine but also with a personalized analysis of every single patient. Today technology is under service of science, and we have to be aware of that.

The concept of “molecular allergy” is not only to request the laboratory technician for determinations of sIgE by sophisticated methods, but also to understand these techniques and apply all this knowledge to benefit our patients. The usage of allergen-based diagnostic methodologies must reach the patient and not only remain for investigation.

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