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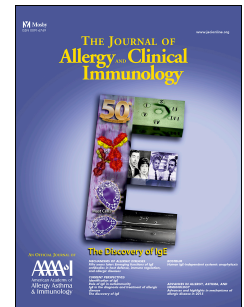
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ImmunoCAP Assays: pros and cons in allergology

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Abstract

Allergen-specific IgE measurements together with the clinical history are the cornerstones of allergy diagnosis. During the past decades not only characterization and standardization of allergen extracts have improved but also assay technology. Here we discuss the uses, advantages, misinterpretations and limitations of ImmunoCAP IgE assays (Thermo Fisher Scientific/Phadia, Uppsala, Sweden) in the field of allergology. They can be performed as singleplex (ImmunoCAP) and since a decade also as multiplex (ISAC). The major benefit of ImmunoCAP is the obtained quantified allergen-specific IgE antibody level and the lack of interference from allergen-specific IgG antibodies. However, ImmunoCAP allergen extract are limited to the composition of the extract. The introduction of allergen molecules has had major impact on the analytical specificity and allergy diagnosis. They are utilized in both singleplex ImmunoCAP and multiplex ImmunoCAP ISAC assays. The major advantage of ISAC is the comprehensive IgE pattern obtained with a minute amount of serum. The shortcomings are its semiquantitative measurements, lower analytical sensitivity and cost per assay. With respect to assay performance ImmunoCAP allergen extracts are good screening tools, but allergen molecules dissect the IgE response on a molecular level and put allergy research on the map of precision medicine.

Fifty years have passed since the discovery of immunoglobulin E and we are not only celebrating IgE but also the first laboratory test for allergy diagnosis, the RAST (radioallergosorbent test).¹ The development of this assay allowed the first reliable identification of allergen-specific IgE. Since then *in-vitro* determination of allergen-specific IgE together with an accurate clinical history of allergy have been cornerstones of the fields of allergy research and clinical diagnosis. During the past decades not only characterization and standardization of allergen extracts have improved but also the range of allergen sources have increased. In parallel, developments in assay technology have improved laboratory aspects such as automation and sample throughput. However, the most important contribution to the field of allergy diagnostics has been the introduction of allergenic molecules/components.² They have opened up a new horizon in allergy research allowing the target for the humoral response to be dissected on a molecular level.

ImmunoCAP assays (Thermo Fisher Scientific/Phadia, Uppsala, Sweden) are the next generation of RAST. The allergen assays can be performed as singleplex (ImmunoCAP) and since a decade also as multiplex (ImmunoCAP ISAC; Immuno Solid-phase Allergen Chip).³ The singleplex assay determines IgE against a single chosen allergen analyte while the multiplex utilizes a fixed array of 112 recombinant or purified native allergen components derived from over 50 allergen sources. Although ImmunoCAP assays also allow allergen-specific IgG and IgG4 measurements, this will not be covered in this article. Here we discuss the uses, advantages, misinterpretations and limitations of the two different ImmunoCAP IgE assays in the field of allergology. There are other IgE assay systems on the market e.g., Immulite (Siemens Healthcare Diagnostics), HYTEC 288/Falcon (Hycor Biomedical) and Microtest allergy system (Microtest Diagnostics) that are beyond the scope of this article. IgE sensitization can also be determined by skin prick test (SPT) using allergen extracts. However, in the European Union allergen extracts for *in vivo* usage have

76 been classified as medical products. Due to the resulting number of quality control
77 requirements certain extracts which do not meet these quality standards are no longer
78 available and additional allergen extracts for SPT are at risk of withdrawal from the market.⁴
79 This makes *in vitro* allergy diagnosis, especially with the development of molecular allergy
80 diagnosis, increasingly important.

81 The singleplex ImmunoCAP assays are based on the binding of allergen-specific
82 IgE antibodies in serum or other body fluids to allergen (extract or allergen molecule) coupled
83 in excess to a solid phase. Bound IgE is subsequently detected using enzyme-labelled anti-
84 human IgE, where the fluorescence intensity is proportional to the amount of allergen-specific
85 IgE. The major benefit of the singleplex ImmunoCAP is the obtained quantified allergen-
86 specific IgE antibody level (kU_A/l), based on the total IgE calibration system traceable to the
87 World Health Organization human reference preparation. In addition, the excess of allergen
88 immobilized on the large cellulose derivative surface of an singleplex ImmunoCAP allows
89 complete binding of IgE antibodies, resulting in high sensitivity and a wide linear assay range.
90 The assay has a good precision and reproducibility and reports down to 0.1 kU_A/l (range 0.1 -
91 100 kU_A/l). Sera with very high IgE antibody levels, >100 kU_A/l, can be accurately quantified
92 using dilutions and subsequent multiplication of the results. In addition, there is no
93 interference from allergen-specific IgG antibodies. All these aspects increase the accuracy of
94 IgE determinations (Figure).

95 In the field of allergy research, a majority of studies are using the singleplex
96 ImmunoCAP allergen extract based assay as it is a good screening tool and the most common
97 method used worldwide, facilitating study comparisons. However, allergen extracts are
98 composed of many different allergens/antigens, some of which have little or no clinical
99 relevance, e.g. carbohydrate epitopes in timothy grass pollen and peanut, which may give
100 irrelevant positive results.⁵ The utility is also impaired in studies focusing on food allergy as

allergen extracts cannot single out the molecular target of the IgE reactivity. This is an important aspect in e.g. peanut allergy where there are different phenotypes based on the IgE response, which is not reflected in the IgE level to peanut extract. One phenotype is associated with no, mild or local symptoms and sensitization to clinically irrelevant carbohydrate epitopes and/or PR-10 proteins causing only local symptoms, and the second is associated with severe systemic allergic reactions and IgE reactivity to storage proteins.⁶ Furthermore, some allergen molecules may be in low abundance or even missing in allergen extracts giving false negative results. To overcome the latter pitfall allergen extracts have been spiked with molecules of small natural abundance. Moreover, in patients sensitized to several pollens and with unclear symptoms ImmunoCAP allergen extracts are unable to identify the relevant allergen(s) for allergen-specific immunotherapy (AIT) which impair the outcome of the AIT-studies.⁷ Thus knowledge regarding the ImmunoCAP assay(s) to be used is needed.

Undoubtedly, the most significant scientific and clinical impact on the use of ImmunoCAP assays has been the introduction of allergen molecules.^{2, 8} They are available from most important allergen sources, either as recombinant expressed proteins or as native molecules purified from natural sources and are utilized in both singleplex ImmunoCAP and multiplex ImmunoCAP ISAC assays. Numerous studies have demonstrated the successful use of allergen molecules on suspected respiratory, venom and food allergies.⁸ Both assays offer allergen molecules that greatly improve the analytical specificity and diagnosis by identifying molecules that serves as 1) marker allergens found only in specific allergen sources e.g. Fel d 1 (cat), Can f 5 (dog), Phl p 1 and Phl p 5, (timothy grass pollen), Ves v 1 and Ves v 5 (yellow jacket), Api m 1 (honey bee), and confirm genuine (species-specific) sensitization and 2) allergen molecules that are present in several allergen sources and reflect serological cross-sensitization e.g. serum albumins, PR-10 proteins, profilins and polcalcins, cross-reactive carbohydrates.⁸ Thus for AIT studies, tools are now available to determine whether a single, a

few closely related or several widely allergen sources are needed, a usefulness which has been confirmed in a number of studies.⁸ As a consequence, diagnostic algorithms based on molecular diagnosis have been developed for several respiratory and venom allergies.⁸ In food allergy research, molecular allergy diagnostics has allowed to predict type and severity of symptoms which will decrease the need for provocation testing, even though placebo controlled food challenges which are currently considered as a golden standard may be needed for confirmation.⁸ In this regard, a negative result to a risk allergen molecule is of particular importance. The limitations of singleplex allergen molecule assays in allergy research, compared to the multiplex ISAC, are the availability of fewer allergen molecules, the incomplete IgE reactivity profile obtained with single or a few tests, the costs if several measurements are needed and the requirement of a larger amount of serum (Table I).

The multiplex ImmunoCAP ISAC is the most complete platform currently available for simultaneously measuring IgE antibodies against more than 100 allergen molecules.^{3, 9} The assay principle is similar to singleplex ImmunoCAP, but here the various allergen molecules are spotted in small quantities in triplets on a polymer coated glass slide, instead of one allergen which is immobilized in a large cellulose matrix. Allergen-bound IgE antibodies are detected by labeled anti-human IgE and the fluorescence is measured using a laser scanner (Fig 1). The assay has recently been evaluated by different operators in 23 sites worldwide. Essentially the same results were obtained regardless of assay site, laboratory-specific conditions, operator, and batches of the microarray and the variability across the whole study was estimated at 25.5%.⁸ The minute amount of serum required for ISAC (40 µl) is a great advantage. This has been of major importance in the European Union-funded project Mechanisms of the Development of Allergy (MeDALL), where an extended chip based on the ISAC technology was developed for studying changes in the allergic immune responses and development of allergic disease in birth cohorts with limited availability of sera.¹⁰ These

studies have identified risk molecules in childhood (e.g. Bet v 1, Fel d 1, and Can f 1) for the development of respiratory disease in adolescence.^{8, 11} In addition, IgE signatures to peanut storage allergens in early childhood that allow predicting course and symptoms of peanut allergy in adolescence have been pinpointed.⁶ Finally, allergen microarrays may prove useful for monitoring the effects of AIT as they take the induction of blocking (allergen-specific IgG) antibodies into account.¹² Thus, IgE levels measured by microarray should better reflect the potency of IgE to mediate allergic reactions.¹⁰ The shortcomings of ISAC are its semiquantitative measurements (values reported in ISU), its lower analytical sensitivity (cut-off 0.3 ISU) and the cost per assay which limits its use in allergy research (Table). Furthermore, all allergen sources are not yet completely represented on the array. One thing to consider when using multiplex testing is handling of at the time clinically irrelevant positive results. However, these molecules may have value in predicting future allergic symptoms. Finally, the increasing experience of molecular allergology, irrespective of singleplex or multiplex ImmunoCAP assays, has resulted in many reports regarding guidance in usage, interpretation and limitations of molecular-based allergy diagnostics.⁸

Future demands for improving IgE assays are to ensure that all important allergen molecules are available in extract based assays (standardized allergen extracts), to broaden the panel of available allergen molecules (correctly folded molecules), to increase and/or diversify the number of allergens in multiplex arrays and to increase the sensitivity of multiplex chip-based assays. This will help address issues such as diagnosing allergies in patients like small children with low IgE levels and evident clinical symptoms.

Allergen-specific IgE measurements together with the clinical history have since decades been fundamental in the diagnosis of allergic disease. With respect to assay performance in allergy research, singleplex assays give maximum sensitivity, while multiplex assays provide a comprehensive IgE pattern with a minute amount of serum. ImmunoCAP

allergen extracts are good screening tools due to their comprehensive content (which can have both positive and negative effects pending on per protein abundance). However, the use of allergen molecules will not only dissect the IgE response on a molecular level, but also put allergy research on the map of precision medicine.

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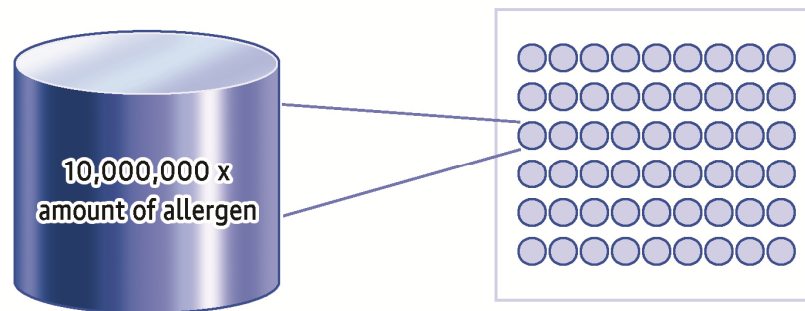
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TABLE I. ImmunoCAP singleplex vs multiplex

ImmunoCAP	ImmunoCAP ISAC
Quantitative results, IgE values tracable to WHO reference preparation	Semiquantitative results
High sensitivity per allergen	Broad sensitization profile
No interference with IgG	Interference with IgG
A selection of crude extracts and/or allergen molecules from many sources	A fixed selection of 112 highly defined proteins (allergen molecules)
One result	112 results
50 µl serum per one allergen/assay	40 µl serum per 112 allergens/assay
Cost advantage in small screening	Cost advantage in large screening
Worldwide experience and usage	Less frequently used, comparably demanding in result interpretation and assay quality control
<i>Extract-specific caveats:</i>	
Detection of low affinity antibodies with little or low clinical relevance	
Allergens in low abundance in extracts may lead to low sensitivity	

Figure 1 legend:

A brief comparison of the single and multiplex ImmunoCAP assays

Fig. 1 The ImmunoCAP assays**ImmunoCAP****ImmunoCAP ISAC**

Allergen:	Allergen extracts or molecules	Allergen molecules
Amount of allergen:	1-2 µg per ImmunoCAP	100 fg / spot
Number of allergens:	One per ImmunoCAP (one allergen source/ mix (around 10 allergen sources)/allergen molecule	112 per chip
Amount of serum:	50 µl per ImmunoCAP	40 µl per ISAC chip
Sensitivity:	0.1 kU/L (calibrated to WHO human reference preparation)	0.3 ISU
Reported answer:	One per ImmunoCAP	112 per chip
Interference with IgG:	No	Yes
Coefficient of variation:	Low	Comparably greater