Molecular Allergy Diagnostics Using Multiplex Assays

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9.1 Introduction

Since Charles Blackley carried out the first in vivo test with pollen on his own skin in 1880 (Blackley 1880), the diagnosis of type I allergy has been performed using extract preparations. Almost 90 years later, shortly after the discovery of immunoglobulin E (IgE), the radioallergosorbent test (RAST) was established. This test enabled circulating specific IgE (sIgE) antibodies to be determined for the first time in vitro, using anti-IgE antibodies labeled with radioisotope (Ishizaka and Ishizaka 1967; Johansson and Bennich 1967; Wide et al. 1967). IgE binding to allergen extracts coupled to a solid phase (paper discs) was measured. The elucidation of the major birch pollen antigen Bet v 1 deoxyribonucleic acid (DNA) sequence heralded the era of molecular allergy diagnostics (Breiteneder et al. 1988). Recombinant or purified (glyco-)proteins enabled the measurement of sIgE to defined single allergens—initially in singleplex and, since 2001, also in multiplex assays (Valenta and Kraft 2001, Hiller et al. 2002) (◉ Fig. 9.1).

Multiplex assays in allergy diagnostics refer to the simultaneous determination of sIgE to different allergens or allergen extracts in a single test run. This approach has already been used in the past in the form of strip tests for allergy screening (e.g., Allergodip, Euroline, Polycheck, etc.), in order to obtain as much information as possible on the sensitization status of an allergic patient in a single test.

These strip tests are based on the "dot blot" principle, in which multiple dot-shaped or strip-shaped allergen-containing membranes serve as the solid phase. These tests enable simultaneous semiquantitative measurement of sIgE to different allergen sources; they do not, however, enable elucidation of the sensitization pattern on a molecular level, since extracts are usually used.

Definitions

Allergen (also single allergen or allergen components)	Molecule with the ability to bind sIgE or trigger sIgE production
Allergen source	Organism that expresses allergenic molecules (e.g., cat, grass pollen)
ISAC	Immuno Solid-phase Allergen Chip, multiplex tool for the determination of sIgE using microarray technology
Microarray	Term used for molecular biological test methods that allow parallel testing of multiple analytes (also known as bio- or allergen chip)
Multiplex assay	Simultaneous testing of multiple analytes in a single assay (e.g., using microarray technology)
Singleplex assay	Testing of a single analyte in a single assay
Diagnostic sensitivity	The probability that a test yields a positive result in an affected individual
Diagnostic specificity	The probability that a test yields a negative result in a healthy individual
Coefficient of variation	Measure of relative dispersion

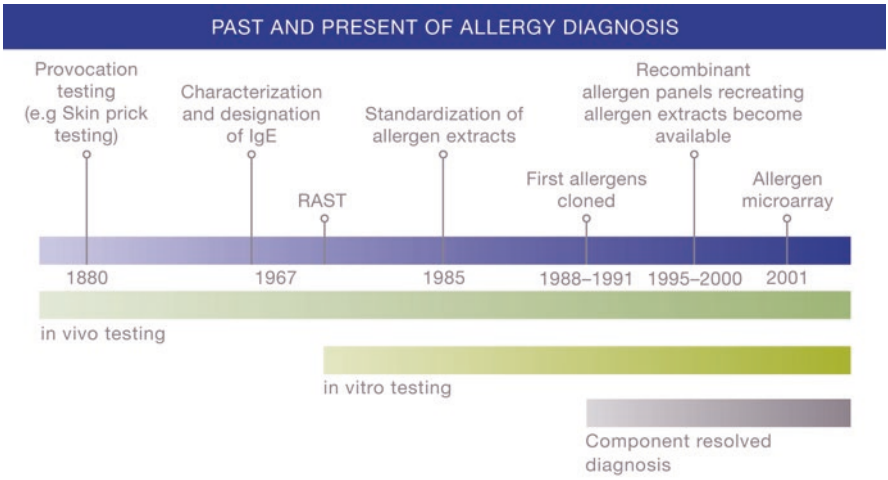

Fig. 9.1 Historical development of diagnostics in IgE-mediated allergies

Thanks to the progress made in molecular allergology and chip-based microarray technology, multiplex assays could be developed which renders the analysis of a patient's IgE profile at the level of individual molecules possible. To accomplish this, minute quantities (picogram range) of different allergens are coupled to a solid phase before these protein arrays (allergen chips) are used for simultaneous determination of allergen-specific IgE (Hiller et al. 2002). In contrast to single tests (singleplex assays) and extract-based diagnostics, allergen chips enable elucidation of an extensive sensitization profile at the individual molecule level in a single measurement. This enables a differentiated analysis of the individual IgE repertoire and reveals a patient's current sensitization status.

The present chapter first introduces the multiplex diagnostic procedure. It then goes on to discuss the advantages and limitations of this new technology for allergy diagnostics in clinical routine and in the research environment.

9.2 Molecular Allergy Diagnostics Using Multiplex Assays

Whereas singleplex assays for molecular allergy diagnostics are already used by and available from many manufacturers of diagnostic tools, there are currently only a few companies with multiplex assays for molecular allergy diagnostics at their disposal.

Of these test systems, one has established itself as the gold standard in multiplex assay molecular allergy diagnostics. This system is based on the Immuno Solid-phase Allergen Chip (ISAC), which has been available since 2001. The ISAC was initially developed and manufactured by VBC Genomics in Vienna; since 2009, it has been further developed, manufactured, and marketed by Phadia, Thermo Fisher Scientific, Uppsala, Sweden. Under the product name ImmunoCAP ISAC 112, the current version of this allergen chip enables determination of sIgE to 112 different single molecules from 51 different plant and animal allergen sources (see ◉ Table 9.1 for a detailed list of the allergens used in ISAC 112).

In addition, test systems exist that couple "classic" allergen extracts onto chips for a microarray assay or combine a panel of defined single allergens with extracts. One of these is a test system only recently CE certified for extract- and component-based diagnostics (ADAM, Microtest Diagnostics Ltd, London, UK). This fully automated test system can semiquantitatively determine sIgE to common aero- and food allergens within 4 h. The test principle is based on a protein microarray currently featuring 22 allergen extracts, three recombinant proteins (rBet v 1, rAra h 2, and rCor a 1), and one purified single allergen (nGal d 1). Since virtually no technical or clinical data on the evaluation of the system are hitherto available (Palomba et al. 2014), it is not possible at present to make any statements on test performance.

Another multiplex test system is currently being developed by Abionic. This system is also based on a fully automated microarray assay and enables measurement of sIgE reactivity to common single allergens in different screening panels, e.g., a screening panel with the food and inhalant allergens Gal d 1, Bos d 5, Ara h 2, Bet v 1, Bet v 2, Phl p 1, Phl p 5, Der p 1, Can f 1, and Fel d 1. The system is conceived as a point-of-care instrument (PoC), uses capillary blood, and—according to the manufacturer's data—enables the determination of sIgE within 20 min. There are currently no study data available on this system.

Semiquantitative multiplex testing with allergen extracts using line blot-based paper strips developed by Euroimmun has recently been complemented with allergenic molecules. Several panels are available with 6–14 purified and/or recombinant food and/or inhalant allergens, including an additional CCD marker:

A. *Peanut panel* with recombinant Ara h 1, Ara h 2, Ara h 3, Ara h 5, Ara h 6, Ara h 7, Ara h 9, and Bet v1.
B. *Cow's milk panel* with native Bos d 4, Bos d 5, Bos d 6, and Bos d 8, and cow's milk extract.
C. *Pediatric panel* with rAra h 1, rAra h 2, rAra h 3, rAra h 9, nGal d 1, nGal d 2, nGal d 3, nGal d 4, nBos d 4, nBos d 5, nBos d 6, nBos d 8, plus 2 native cow's milk extracts and rBet v 1.
D. *Pollen panel* with recombinant Bet v 1, Bet v 2, Bet v 4, Bet v 6, Phl p 1, Phl p 5, Phl p 7, Phl p 12, plus birch and timothy pollen extracts.
E. *Insect venom panel* with recombinant Api m 2, Api m 10, Ves v 1, Ves v 5, plus native bee and wasp venom extracts.

So far no published results exist regarding technical or clinical evaluations, making it difficult to conclude on the performance characteristics of these assays.

Most recently, a multiplex test called FABER (version 244-122-122-01) was announced by MacroarrayDX for simultaneous detection of, i.e., allergen-specific IgE to 112 allergenic molecules and 112 extracts. The present custom-developed panel covers reagents allowing simultaneous antibody detection to foods from

Table 9.1 Allergen spectrum in the ImmunoCAP ISAC 112

Allergen source	Allergen	Protein family/biochemical name
Food allergens: *plant*		
Apple	rMal d 1	PR-10
Buckwheat	nFag e 2	2S albumin
Cashew nut	rAna o 2	Cupin
Peanut	rAra h 1	Cupin
	rAra h 2	2S albumin
	rAra h 3	Cupin
	nAra h 6	2S albumin
	rAra h 8	PR-10
	rAra h 9	nsLTP
Hazelnut	rCor a 1.0401	PR-10
	rCor a 8	nsLTP
	nCor a 9	Cupin
Kiwi	nAct d 1	Cysteine protease
	nAct d 2	Thaumatin-like protein
	nAct d 5	Kiwellin
	rAct d 8	PR-10
Brazil nut	rBer e 1	2S albumin
Peach	rPru p 1	PR-10
	rPru p 3	nsLTP
Celery	rApi g 1	PR-10
Sesame	nSes i 1	2S albumin
Soybean	rGly m 4	PR-10
	nGly m 5	Cupin
	nGly m 6	Cupin
Walnut	rJug r 1	2S albumin
	nJug r 2	Cupin
	nJug r 3	nsLTP
Wheat	rTri a 14	nsLTP
	rTri a 19	ω-5-Gliadin
	nTri a aA_TI	α-Amylase/trypsin inhibitor
Food allergens: *animal*		
Cod	rGad c 1	Parvalbumin
Hen's egg	nGal d 1	Ovomucoid
	nGal d 2	Ovalbumin
	nGal d 3	Conalbumin
	nGal d 5	Serum albumin

(continued)

Table 9.1 (continued)

Allergen source	Allergen	Protein family/biochemical name
Cow's milk	nBos d 4	α-Lactalbumin
	nBos d 5	β-Lactoglobulin
	nBos d 6	Serum albumin
	nBos d 8	Casein
	nBos d-lactoferrin	Transferrin
Shrimp	nPen m 1	Tropomyosin
	nPen m 2	Arginine kinase
	nPen m 4	Sarcoplasmic calcium-binding protein
Pollen allergens		
Maple-leaved plane	rPla a 1	Invertase inhibitor
	nPla a 2	Polygalacturonase
	rPla a 3	nsLTP
Arizona cypress	nCup a 1	Pectate lyase
Spreading pellitory	rPar j 2	nsLTP
Ragweed	nAmb a 1	Pectate lyase
Birch	rBet v 1	PR-10
	rBet v 2	Profilin
	rBet v 4	Polcalcin
Annual mercury	rMer a 1	Profilin
Alder	rAln g 1	PR-10
Common mugwort	nArt v 1	Defensin-like protein
	nArt v 3	nsLTP
Hazel pollen	rCor a 1.0101	PR-10
Bermuda grass	nCyn d 1	Grass group 1
Japanese cedar	nCry j 1	Pectate lyase
Timothy grass	rPhl p 1	Grass group 1
	rPhl p 2	Grass group 2/3
	nPhl p 4	Berberine bridge enzyme
	rPhl p 5	Unknown
	rPhl p 6	Unknown
	rPhl p 7	Polcalcin
	rPhl p 11	Ole e 1-related protein
	rPhl p 12	Profilin
Olive tree	rOle e 1	Olive group 1
	nOle e 7	nsLTP (putatively)
	rOle e 9	1,3-β-Glucanase
Prickly saltwort	nSal k 1	Pectin methylesterase
Ribwort plantain	rPla l 1	Ole e 1-related protein
White goosefoot	rChe a 1	Ole e 1-related protein

Table 9.1 (continued)

Allergen source	Allergen	Protein family/biochemical name
Furry animal allergens		
Dog	rCan f 1	Lipocalin
	rCan f 2	Lipocalin
	nCan f 3	Serum albumin
	rCan f 5	Arginine esterase
Cat	rFel d 1	Uteroglobin
	nFel d 2	Serum albumin
	rFel d 4	Lipocalin
Mouse	nMus m 1	Lipocalin
Horse	rEqu c 1	Lipocalin
	nEqu c 3	Serum albumin
Mite allergens		
Blomia tropicalis	rBlo t 5	Unknown
D. farinae	nDer f 1	Cysteine protease
	rDer f 2	NPC2
D. pteronyssinus	nDer p 1	Cysteine protease
	rDer p 2	NPC2
	rDer p 10	Tropomyosin
Lepidoglyphus destructor	rLep d 2	NPC2
Mold allergens		
A. alternata	rAlt a 1	Unknown
	rAlt a 6	Enolase
A. fumigatus	rAsp f 1	Mitogillin
	rAsp f 3	Peroxisomal protein
	rAsp f 6	Manganese superoxide dismutase
C. herbarum	rCla h 8	Mannitol dehydrogenase
Latex allergens		
Latex	rHev b 1	Rubber elongation factor
	rHev b 3	Small rubber particle protein
	rHev b 5	Unknown
	rHev b 6.01	Hevein precursor
	rHev b 8	Profilin
Insect venom allergens		
Common wasp	rVes v 5	Antigen 5
Honey bee	rApi m 1	Phospholipase A_2
	nApi m 4	Melittin
European paper wasp	rPol d 5	Antigen 5
Other allergens		
Pineapple	nMUXF3	Cross-reactive carbohydrate determinants (CCD)

(continued)

Table 9.1 (continued)

Allergen source	Allergen	Protein family/biochemical name
German cockroach	rBla g 1	Unknown
	rBla g 2	Aspartic protease
	rBla g 5	Glutathione S-transferase
	nBla g 7	Tropomyosin
Herring worm	rAni s 1	Unknown
	rAni s 3	Tropomyosin

nuts, seeds, and legumes ($n = 46$); fruits ($n = 31$); vegetables ($n = 13$); milk ($n = 12$); egg and fowl ($n = 15$); meats ($n = 6$); fish, shellfish, and mollusks ($n = 17$); or inhalant allergen carriers, i.e., tree pollen ($n = 13$), grass pollen ($n = 8$), weed pollen ($n = 8$), mites ($n = 10$), epidermal and other animal proteins ($n = 24$), microorganisms ($n = 11$), insects ($n = 7$), and additional allergen sources like insect venoms ($n = 5$), parasites ($n = 5$), latex ($n = 10$), as well as 3 CCD-markers. The allergen reagents are (a) bound to chemically activated nanoparticles allowing individual optimization of the antigen, (b) arrayed to a solid-phase matrix, (c) to form a single-step multiplex test solution for 100 µl of serum or plasma, (d) and finally assayed and quantified by colorimetric or luminescence image capture. Up to now technical data regarding performance characteristics or clinical evaluations are not yet available.

9.3 Immuno Solid-Phase Allergen Chip (ISAC)

9.3.1 Test Procedure

The ImmunoCAP ISAC 112, a solid-phase immunoassay, comprises a polymer-coated slide with four fields, the protein microarrays (i.e., allergen chips) (◉ Fig. 9.2). One array is used per patient sample, such that four different sera can be tested with each slide. The allergens (in the picogram range) are applied in triplicates, thus enabling multiple measurements, and covalently bonded to the polymer layer. The allergen components immobilized in this way bind all allergen-specific antibodies (e.g., IgE, IgG, IgA) in the patient sample (◉ Fig. 9.3). Once the nonspecific antibodies have been washed away, a fluorescently labeled antihuman IgE antibody is added to promote complex formation. Following incubation, unbound antibodies of other isotypes (IgG, IgA, etc.) and excess unbound fluorescently labeled antihuman IgE antibodies are removed by washing. Finally, fluorescence is measured using a microarray scanner (◉ Fig. 9.4). The higher the signal, the more sIgE is present in the sample. The test results are analyzed with PC-based software, and the concentration of sIgE in the sample is calculated in the form of ISAC standard units (ISU-E). The manufacturer has adjusted the calibration curve to approximately match the units in the ImmunoCAP singleplex method (kU_A/l). The latter are derived heterologously over a total IgE standard curve, whereas ISU-E are based on calibration using the ImmunoCAP singleplex system (Phadia 250).

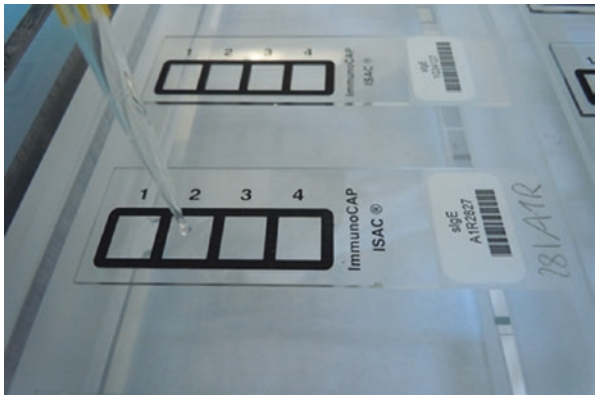

Fig. 9.2 ISAC Allergen chip: example of a commercially available multiplex assay to simultaneously measure sIgE to 112 single allergens

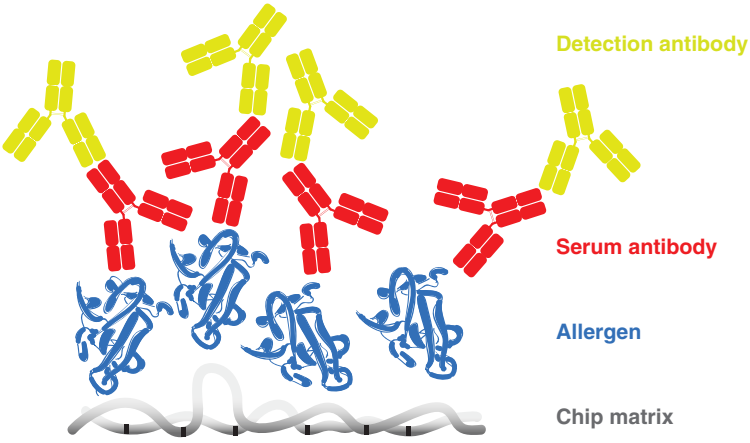

Fig. 9.3 Test principle of the ISAC allergen chip

Measurement values are reported not only quantitatively but also semiquantitatively, divided into four different categories:

1. Values <0.3 ISU-E are defined as negative.
2. Values between 0.3 and 1.0 ISU-E as low-level positive.
3. Values between 1.0 and 15.0 ISU-E as moderately high.
4. Values ≥15.0 ISU-E as very high.

Thus, test results comprise the actual measurement, plus a color-coded bar chart representation from which the approximate value of the measurement and the evaluation category can be read.

The ISAC 112 is primarily defined as a semiquantitative method, since, in the manufacturer's opinion, the miniaturization of the assay design, the shape of the calibration curve, the degree of scattering, and potential divergent values due to competitive inhibition by competing allergen-specific antibodies of other classes (see below) preclude reliable measurement of the "true" quantitative concentrations of allergen-specific IgE antibodies.

Fig. 9.4 Example of ISAC 112 microarray analysis with triplicate measurements of sIgE signals

9.3.2 Test Performance

Extensive data on test performance were collected for ISAC 112 by the manufacturer in 2011 (ImmunoCAP ISAC 112—performance characteristics, data on file, 2011) and relate to the following parameters:

- Precision (reproducibility depending on signal strength)
- Intra-assay variation coefficients (IAVC) and inter-assay variation coefficients (IEVC)
- Linearity (measurement response using diluted samples)
- Limit of detection (LoD) and limit of quantitation (LoQ)
- Matrix effects
- Total IgE interference
- Parallel comparison with singleplex tests (ImmunoCAP)

Data on precision, linearity, and LoD as well as on factors possibly causing interference in the assay are discussed in the following sections.

9.3.2.1 Intra- and Inter-Assay Variance

Data on precision were collected using sera from four multisensitized patients. The samples were measured in triplicate a total of 17 times over a 4-week period. This approach generated data on intra- and inter-assay variance for 105 of 112 allergens.

According to the manufacturer, the average coefficient of variation (CV) for all allergens tested in intra- and inter-assay comparisons is below 20 %. However, it should be noted that the CV values change depending on the test system's measurement range (0.3–1.0 ISU-E vs. 1.0–15 ISU-E vs. >15 ISU-E), with higher values reported in the lowest measurement range (◉ Fig. 9.5, ◉ Tables 9.2 and 9.3).

9.3.2.2 Linearity and Limit of Detection (LoD)

Investigations of linearity were performed using serial 1:2 dilutions on sera with high sIgE values (>5 ISU-E) to the respective allergen. In this manner, linearity curves and regression coefficients were calculated for 81 of the 112 allergens, which confirmed the linearity between measurement values and orders of dilution in wide ranges (◉ Fig. 9.6 and ◉ Table 9.4).

The LoD (▶ Chap. 7), defined as the lowest sIgE concentration that can be reliably determined, was determined for eight representative allergens (Ara h 1, Bet v 1, Der p 1, Equ c 1, Fel d 1, Gad c 1, Gal d 1, and Phl p 5) according to the global consensus on the standardization of healthcare technology guidelines (NCCLS-EP17-A). The LoD was between 0.05 and 0.28 ISU-E for the individual allergens. Based on these results, and considering the identical test conditions and known CV values in the lowest measurement range, an LoD of <0.3 ISU-E was arrived at for all 112 allergens. However, according to the manufacturer, sIgE concentrations <1 kU_A/l are

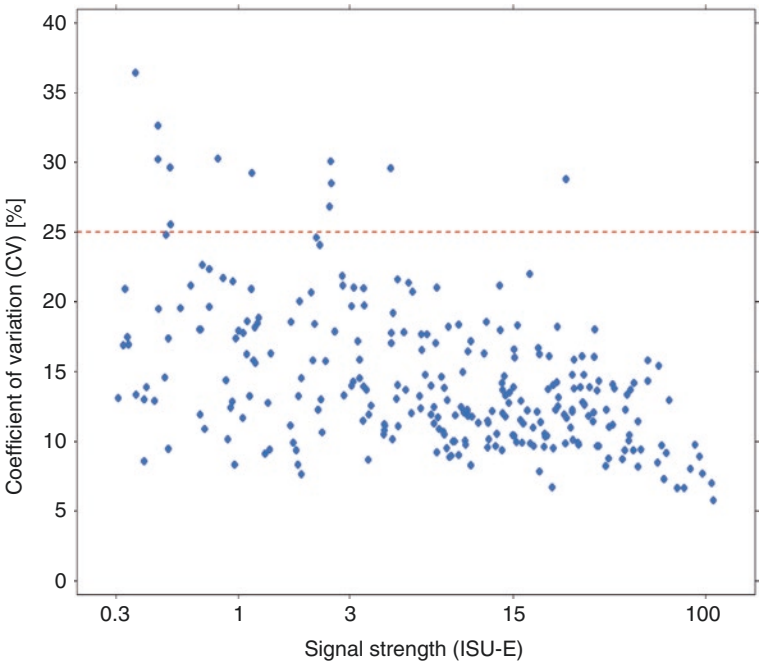

Fig. 9.5 Coefficient of variation (CV) depending on signal strength (ISU). Four serum samples covering 105 single allergens were used for calculation. Each sample was analyzed in triplicate in a total of 17 runs over a 4-week period (From "ImmunoCAP ISAC 112—performance characteristics," data on file, 2011; used with permission from Thermo Fisher Scientific)

Table 9.2 Representative examples of coefficients of variation for sIgE measurements against single allergens depending on signal strength

Sample	Allergen	Signal strength ISU-ISU-E	Mean ISU-E	CV intra-assay variance (%)	CV inter-assay variance (%)
1	Par j 2	0.33–0.98	0.32	18	9
2	Gal d 1		0.46	11	16
3	Cry j 1		0.98	12	13
4	Equ c 1	1.2–14	1.2	15	11
5	Der f 1		4.6	5	9
6	Fel d 1		14	8	9
7	Ara h 1	19–90	19	11	13
8	Phl p 5b		47	6	7
9	Bet v 1		90	7	7

CV coefficient of variation

Table 9.3 Averaged coefficient of variation for all allergens depending on signal strength

ISU-E	Class	CV intra-assay variance (%)	CV inter-assay variance (%)
0.3–1	Low	7	14
1–15	Moderate	6	10
>15	High	5	9

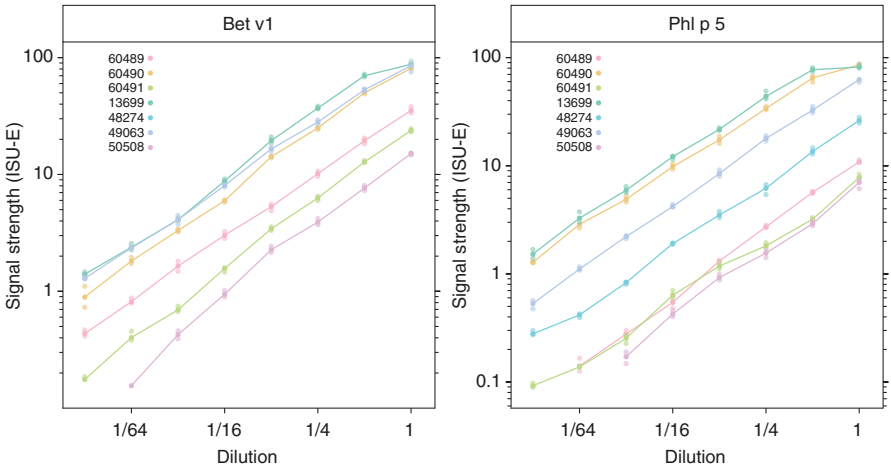

Fig. 9.6 Linearity of measurements in a dilution series using the examples of Bet v 1 and Phl p 5: serial 1:2 dilutions of seven different sera with sIgE values >5 ISU/l (From "ImmunoCAP ISAC 112—performance characteristics," data on file, 2011; used with permission from Thermo Fisher Scientific)

Table 9.4 Representative data on linearity (slope) and regression coefficient (R^2) of different allergens

Allergen	Slope	R^2
Ara h 2	1.03	0.96
Ber e 1	1.07	0.97
Bet v 1	1.16	0.95
Can f 1	1.12	0.92
Cyn d 1	1.09	0.91
Der f 2	1.01	0.99
Equ c 1	1.18	0.93
Gal d 1	1.01	0.99
Pen m 1	1.07	0.97
Phl p 1	1.12	0.97

not reliably detectable by the ISAC 112 system. Therefore, the overall sensitivity (LoD, LoQ) of ISAC 112 is to be considered lower than that of the ImmunoCAP (singleplex) method.

9.3.2.3 Sample Material and Interference

Investigations comparing sample materials were carried out on serum, citrate, heparin, and ethylenediaminetetraacetic acid (EDTA) plasma from identical donors and showed that serum, citrate, or heparin plasma from capillary or venous blood can be used. Using EDTA plasma can cause interference with Ca^{++}-binding allergens (e.g., Gad c 1, Pen m 4, or polcalcin Bet v 4 and Phl p 7) and thus lead to false-negative or false low results. When testing hemolytic or lipemic samples, neither hemolysis (up to 5 %) nor hypertriglyceridemia (triglyceride concentration up to 12 mg/ml) caused significant interference in the test system.

A factor known to influence the determination of sIgE in solid-phase assays is the level of total IgE. In order to test this influence, IgE-negative serum samples and four serum samples exhibiting sIgE to 68 of the 112 allergens were spiked with high total IgE concentrations (3,000 or 10,000 kU/l) and measured in parallel. As shown in ◉ Fig. 9.7, supplementing high concentrations of total IgE had no effect on test performance.

9.3.3 Comparison of sIgE to Single Allergens Determined in Multiplex (ISAC sIgE 112) and Singleplex Assays (ImmunoCAP)

Using 350 sera and 57 allergens that were also available as ImmunoCAP singleplex reagents, the manufacturer compared the two different measurement systems.

Depending on the frequency of sensitization, a correlation of the measured values was demonstrated for each allergen with at least five, maximally 75 sera. As shown by way of example in ◉ Fig. 9.8, a good to very good correlation of the

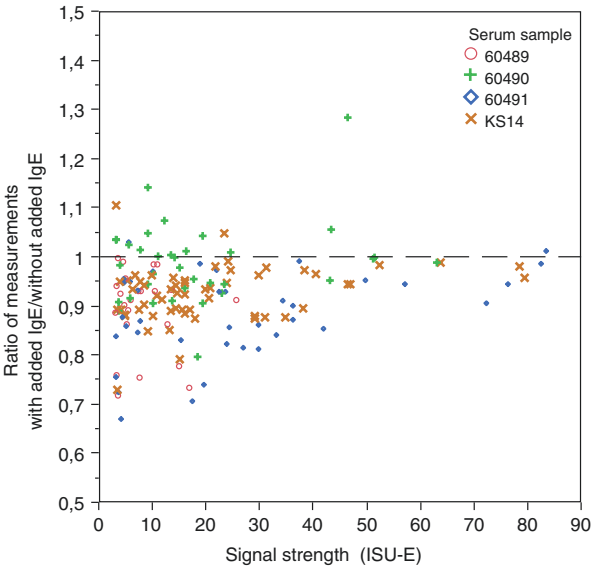

Fig. 9.7 The addition of high concentrations of IgE has no significant effect on measurements. The quotients of the measurement values obtained with and without the addition of IgE (10,000 kU/l), as measured in different serum samples, are shown (From "ImmunoCAP ISAC 112—performance characteristics," data on file, 2011; used with permission from Thermo Fisher Scientific)

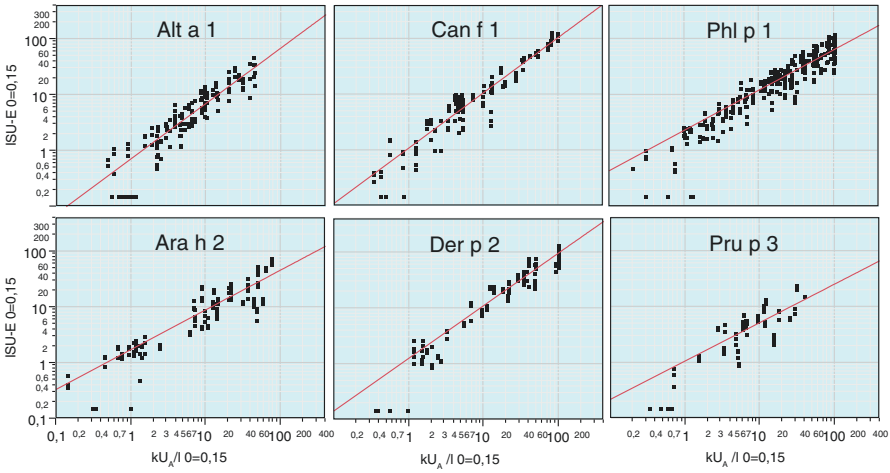

Fig. 9.8 Comparison of measurements made using the ISAC sIgE 112 (ISU-E) and ImmunoCAP (kU_A/l) systems for selected single allergens. Negative results (<0.15) are plotted as 0.15 ISU-E (From "ImmunoCAP ISAC 112—performance characteristics," data on file, 2011; used with permission from Thermo Fisher Scientific)

ISU-E values with the ImmunoCAP-derived values (kUA/l) was observed for many allergens. However, the test sensitivity of ImmunoCAP is clearly higher for some allergens (i.e., LoD is lower). Another investigation used sera from 82 patients and a total of 555 measurements of sIgE to single allergens to compare the two methods (Gadisseur et al. 2011). Using negative cutoff values of <0.3

ISU-E and <0.35 kUA/l (or <0.1 kUA/l), a concordance rate of 92.2 % (or 78.7 %) was found for the positive results. The concordance rate for the negative findings was 93.6 %.

Although excellent concordance rates were seen for most allergens, clear discrepancies were shown for isolated allergens. These included rAsp f 1 (9/14), rPup p 3 (5/13), nAna c 2 (4/11), and rApi g 1 (4/10) (Gadisseur et al. 2011). Differences in the performance of individual allergens can potentially be explained by the differing presentation of allergens on the solid phase of the assay. Compared with immobilization on the polymer coating of the glass chips, covalent binding of allergens to the cellulose matrix in the CAP system can result in different epitopes being exposed or blocked and thus to suboptimal binding of sIgE present in the sample. Additional differences between the set-ups of the two test systems can cause discrepant results in particular cases. Whereas a large excess of allergen is present in the ImmunoCAP system, thus leading to binding of all sIgE present in the sample in most cases, much less allergen is present in the ISAC assay. This can mean that not all allergen-specific IgE will find a binding partner, thus leading to lower results. In this respect, other allergen-specific antibody isotypes (particularly IgG) play a significant role, since these can also block the IgE-binding sites (IgE epitopes), resulting in lower IgE concentrations. On the other hand, the kinetics generated by the large excess of allergen in the ImmunoCAP singleplex assay allow binding of low-affinity sIgE, whereas the kinetics of ISAC 112 ensure that high-affinity sIgE is preferentially bound.

9.4 Molecular Allergy Diagnostics Using Multiplex Assays in Clinical Routine

9.4.1 Allergen Spectrum Available and Potential Advantages in Diagnostics

With 112 individual allergens from 51 allergen sources, the ImmunoCAP ISAC 112 assay currently offers the widest allergen spectrum for molecular allergy diagnostics in clinical routine. Particularly those allergens were selected that:

- Frequently cause sensitizations
- Confer an additional benefit in the interpretation of individual sensitization profiles

The current version of the allergen chip includes:

- 43 single allergens from 17 different foods
- 30 single allergens from 16 different seasonal aeroallergen sources
- 27 single allergens from 13 different perennial aeroallergen sources
- 12 additional single allergens from other allergen sources

Detailed analysis of IgE sensitizations using the allergen chip enables differentiated diagnostics, whereby the advantages of broad molecular screening are evident, even without knowledge of clinical symptoms, from a universal analytical perspective (on the test level). The following consequences or particular arguments should be considered when using these single allergens in microarray format:

A. *Increased test sensitivity* (low limit of quantitation, LoQ) achieved by using specific single allergens compared with diagnostics using allergen extracts
B. *Improved analytical specificity (selectivity)* for particular single allergens with special characteristics (e.g., IgE sensitization associated with severe reactions)
C. *Indicators of cross-reactivity* (common cause of a lack of analytical specificity of allergen extracts)
D. *Markers of primary, genuine* (possibly species-specific) *IgE sensitization*
E. Ideally, *complete representation of the individual sensitization profile* (in contrast to singleplex specific molecular IgE diagnostics)

Criterion A
Individual allergens underrepresented or lacking in an allergen extract can bind sIgE better when used in a specific manner in the microarray, thus generating positive signals and indicating sensitizations more accurately. However, the limit of quantitation (LoQ, ▶ Chap. 7) is usually lower for singleplex methods than it is in microarray, due to the large amounts of (single) allergen used. This explains the limited precision and accuracy of microarray at sIgE concentrations below 1 kUA/l. Therefore, especially sera with low total IgE (<25 kU/l) can yield false-negative values to certain single allergens in the microarray analysis; for this reason, singleplex testing is preferred (to microarray) in such constellations.

Criterion B
Increased analytical specificity is especially desirable when the specific physicochemical characteristics of the single allergens concerned are associated with particular clinical consequences (e.g., high allergen stability and/or high proportion of the total allergen source as the cause of risk-associated sensitizations, e.g., to particular foods; localization of the allergens as a means of differentiating between certain clinical presentations, e.g., sIgE to intracellular *Aspergillus* allergens in bronchopulmonary aspergillosis).

Increased analytical specificity is not an advantage per se—only when the selective information regarding the allergen in an extract is associated with a predefined (clinical) characteristic does this have a significant benefit for molecular diagnostics.

Criterion C
Single allergens improve, in particular, the allergen specificity of IgE sensitization tests. In light of this, certain conserved allergen molecules that are of similar structure, have common IgE-binding epitopes, and occur in numerous allergen sources

have proven useful as indicators for identifying potential cross-reactivity (see also ▶ Chap. 7). They form the basis for concomitant sensitizations to different allergen sources with extremely variable biological relationships.

Criterion D
Other single allergens, in contrast, yield important information regarding a genuine primary IgE sensitization on the basis of:

- Their well-defined, particular structure
- Their IgE epitopes with limited similarity in other single allergens
- Their presence in highly specific allergen sources

Single allergens reestablish the necessary analytical specificity, particularly in the case of allergen sources with known cross-reactive single allergens.

Criteria A–D are by no means mutually exclusive, since single allergens can embody several advantages. Their value in molecular diagnostics (in both single and multiplex assays) varies for each allergen molecule from case to case and must be redefined on the basis of the specific question.

Criterion E
In contrast to singleplex testing, multiplex assays ideally reveal all potential sensitizations. This discloses the entire spectrum of an individual's susceptibility to allergy, and the allergen-specific IgE repertoire can then be systematically checked for possible or absent clinical relevance. This procedure is currently also referred to as a bottom-up approach (in contrast to the top-down approach based on medical history, skin and/or IgE tests with allergen extracts, followed by specific singleplex IgE testing using single allergens).

Examples of the Advantages of Molecular Multiplex IgE Analysis

The following sections provide concrete examples of the generally formulated advantages of molecular multiplex IgE analysis.

Using molecular sensitization profiles, it is possible to differentiate, e.g., primary sensitizations (D) from cross-sensitizations (E), for instance, genuine, primary food allergies from pollen-related, secondary food allergies. These interpretations require comprehensive knowledge of the single allergens, their molecular characteristics, and their affiliation to particular protein families.

The molecular and physicochemical characteristics of single allergens represent a further level on which to base differentiation, e.g., the sensitivity or resistance of food protein to heat and peptic digestion by gastric acids. For example:

- Storage proteins (2S albumins, cupins) are characterized by their strong resistance.
- Profilins and PR-10 are characterized by high sensitivity, respectively, to heat and digestion.

The clinical relevance of the different sensitizations can be illustrated using peanut allergens as an example: sensitization to storage proteins (Ara h 2, Ara h 1, Ara h 3, and Ara h 6) is associated with a significantly increased risk of a systemic reaction following peanut consumption, whereas sensitization to the PR-10 protein from peanut (Ara h 8) is associated with only a low risk, e.g., predominantly oropharyngeal symptoms (Asarnoj et al. 2012).

◉ Table 9.1 provides a detailed list of the single allergens and their affiliation to the different protein families. Important protein families represented on the allergen chip, as well as their main characteristics, are summarized in ◉ Table 9.5.

9.4.2 Added Benefits Conferred by Molecular Allergy Diagnostics in Clinical Routine

9.4.2.1 Differentiation Between Genuine Sensitization and Cross-Reactivity with Inhalant Allergens

In pollen allergy patients exhibiting serological or skin test reactivity to various pollen species (e.g., birch, grasses, mugwort), this may indicate either a genuine sensitization to the particular type of pollen or be caused by IgE cross-reactivity to cross-reactive panallergens, such as:

- Profilins (e.g., Bet v 2, Phl p 12, Art v 4, and Amb a 8)
- Polcalcins (e.g., Bet v 4, Phl p 7, Art v 5, and Amb a 10)

Differentiation between a genuine sensitization and cross-reactivity is only possible if IgE reactivity to specific marker allergens can be demonstrated. Only then does the reactivity result from a genuine primary sensitization to the relevant allergen source. To enable such a distinction to be made, the ISAC 112 assay features numerous marker allergens from different pollen species, including:

- Bet v 1 for birch pollen
- Ole e 1 for ash pollen
- Pla a 1 for plane pollen
- Cup a 1 for cypress pollen
- Phl p 1, Phl p 2, Phl p 5, Phl p 6, and Phl p 11 for grass pollen
- Art v 1 for mugwort pollen
- Amb a 1 for ragweed
- Pla l 1 for ribwort plantain
- Che a 1 for goosefoot

At the same time, the IgE reactivity to panallergens such as profilins (Phl p 12, Bet v 2) and Polcalcins (Phl p 7, Bet v 4) can be determined in order to obtain information on potential cross-reactivity. To what extent panallergens can contribute to allergic reactions and clinical manifestations of pollen allergies is still the subject of debate. However, due to their high degree of cross-reactivity, these panallergens

Table 9.5 Cross-reactive protein families represented in ISAC 112 as well as their main characteristics

Profilins	Sensitive to heat and digestion; tolerance of cooked foods common
	Although rarely associated with clinical symptoms, can cause local and severe reactions in some patients
	Profilins are found in all pollens and plant foods
Polcalcins	Marker for cross-reactivity between different pollen species
	Polcalcins are not found in plant foods
PR-10 proteins (Bet v 1 homologs)	Generally sensitive to heat and digestion; tolerance of cooked foods common
	Generally associated with local symptoms, such as oral allergy syndrome
	Associated with allergic reactions to pollen, fruit, and vegetables
Serum albumins	Sensitive to heat and digestion
	Found in fluids and tissue, e.g., in cow's milk, blood, beef, and dander
	Cross-reactivity between serum albumins from various mammal species, e.g., between cat and dog
Nonspecific lipid transfer proteins (nsLTP)	Resistant to heat and digestion; reactions to cooked foods possible
	Often associated with systemic and severe reactions besides oral allergy syndrome
	Associated with local reactions to fruit and vegetables
	Found in some pollen species (e.g., mugwort)
Tropomyosins	Resistant to heat and digestion; reactions to cooked foods possible
	Often associated as a food allergen with systemic and severe reactions
	Proteins found in muscle fibers, responsible for cross-reactivity between invertebrates (e.g., house dust mite and shrimp)
Lipocalins	Stable proteins and important allergens in furry animals
	Allergens with different cross-reactivity between various furry animals
Storage proteins (2S albumins, cupins)	Resistant to heat and digestion; reactions to cooked foods possible
	Often associated as a food allergen with systemic and severe reactions in addition to OAS
	Found in seeds and nuts, serve as source material for growth of the new plant
Parvalbumins	Resistant to heat and digestion; reactions to cooked foods possible
	Often associated as a food allergen with systemic and severe reactions in addition to OAS
	Major allergen in fish

represent a considerable problem for the detection of allergen-specific sensitization using extract-based methods. For this reason, it is particularly important to perform sIgE diagnostics using species-specific marker allergens in polysensitized patients, alongside a consideration of the precise medical history. These tests yield information relevant to selecting the correct extract prior to commencing immunotherapy.

Diagnostic testing using the ISAC 112 multiplex platform reveals an extensive sensitization profile, including the most common marker and cross-reactive allergens, in a single measurement.

9.4.2.2 Identification of Sensitizations to Food Allergens Associated with a High Risk for Severe Allergic Reactions

IgE to food extracts can be the result of cross-reactivity with pollen-associated allergens, such as allergens of the Bet v 1 or profilin families.

Pollen allergens of the Bet v 1 family include:

- Bet v 1 (birch)
- Aln g 1 (alder)
- Cor a 1 (hazel)
- Que a 1 (oak)
- Fag s 1 (beech)

In the case of relevant sensitization to these aeroallergens, cross-reactivity with the following food allergens is common due to high sequence and structural homology:

- Pome and stone fruits and nuts (hard-shelled fruits), e.g., Act d 8 (kiwi), Cas s 1 (chestnut), Cor a 1 (hazel), Fra a 1 (strawberry), Mal d 1 (apple), Pru p 1 (peach), and Pyr c 1 (pear)
- Vegetables and legumes, e.g., Api g 1 (celery), Ara h 8 (peanut), Dau c 1 (carrot), Gly m 4 (soy), and Vig r 1 (mung bean)

Similarly, it is assumed that sensitization to pollen-mediated profilins can cause cross-reactivity with corresponding profilins in food. The pollen profilins responsible for sensitizations in areas with high grass pollen counts are mainly grass pollen profilins, such as Phl p 12 (timothy grass). Less frequently, Bet v 2 (birch) or Art v 4 (mugwort)—in other regions possibly Amb a 8 (ragweed) or Ole e 2 (olive)—can also be the cause of profilin sensitization.

In terms of food, corresponding profilins are present in fruits, e.g.:

- Ana c 1 (pineapple)
- Cit s 1 (orange)
- Cuc m 2 (melon)
- Fra a 4 (strawberry)
- Mal d 4 (apple)

As well as in legumes and vegetables:

- Ara h 5 (peanut)
- Gly m 3 (soy)
- Api g 4 (celery)

- Cap a 2 (bell pepper)
- Dau c 4 (carrot)
- Lyc e 1 (tomato)

Allergens of the Bet v 1 family and profilin family are sensitive to heat and digestion and generally only cause local oropharyngeal symptoms. Exceptions to this may be observed if large quantities of untreated, "native" allergens are consumed. In the absence of heat treatment or previous processing and denaturation of proteins, systemic reactions may occur. A classic example of this is consumption of native soy milk by individuals highly sensitized to Gly m 4.

In contrast to pollen-associated food allergies to Bet v 1 homologs or profilins, sensitization to food allergens from the storage protein families is frequently associated with a significantly increased risk for severe allergic reactions: storage proteins are extremely resistant to heat and digestion and are present in legumes and tree nuts in large quantities.

A distinction is made between different storage protein families:

- 11S globulins (legumins)
- 7S globulins (vicilins)
- 2S albumins

The following nut storage proteins are characterized:

- Hazelnut: Cor a 9, Cor a 11, and Cor a 14
- Walnut: Jug r 1, Jug r 2, and Jug r 4
- Pecan nut: Car i 1, Car i 2, and Car i 4
- Almond: Pru du 6
- Cashew: Ana o 1, Ana o 2, and Ana o 3
- Pistachio: Pis v 1, Pis v 2, Pis v 3, and Pis v 5
- Brazil nut: Ber e 1 and Ber e 2

Among the legumes:

- Peanut: Ara h 1, Ara h 2, Ara h 3, and Ara h 6
- Soy: Gly m 5, Gly m 6, and Gly m 8

The detection of sIgE to specific storage proteins serves as an indication for an increased risk of severe allergic reactions to small quantities of the allergen. IgE detections to the following allergens are particularly important:

- Ara h 2 in peanut allergy
- Cor a 9 and Cor a 14 in hazelnut allergy
- Jug r 1 and Jug r 4 in walnut allergy
- Ber e 1 in Brazil nut allergy

Similarly, the detection of sIgE to members of the lipid transfer protein (LTP) family appears to be associated with an increased risk of systemic reactions. This includes peach LTP Pru p 3—particularly in patients from Mediterranean regions that have been sensitized cutaneously by the high LTP content of the skin of ripe peaches—as well as walnut Jug r 3 and hazelnut Cor a 8. Since many of the aforementioned allergens are present on the allergen chip, the ISAC 112 multiplex diagnostic test largely reveals individual sensitization profiles and thus forms the basis for risk assessment during subsequent patient counseling.

9.4.3 Paralysis Through Analysis? Interpretation Supported by Intelligent Software and Results Evaluated by the Physician

Using ISAC 112 to simultaneously determine 112 parameters in order to generate a detailed sensitization profile presents a challenge for the physician, particularly in the case of polysensitized patients. The manufacturer's X-plain software integrated into the ISAC 112 system ensures a systematic compilation of positive results in a medical report and simplifies interpretation of the relevance of the detected sensitizations.

Section one of the medical report (▶ e.g., X-plain medical report) relates to general details about whether sensitizations to marker allergens and/or cross-allergens are present and whether IgE reactivity to allergens associated with an increased risk of systemic reactions was found.

Section two includes details on sensitizations to food allergens and aeroallergens. In addition to the IgE reactivities detected, this section provides an aid to interpretation as well as details on the particular features of specific sensitizations, such as regional variations (Ole e 1, the marker allergen for olive pollen, is considered a marker for ash sensitization in areas with high ash populations; Cry j 1, a marker allergen for the Japanese cedar, is considered a marker for sensitization to cypresses).

Section three of the medical report, which describes sensitizations to cross-reactive foods and aeroallergens, also provides interpretation aids and background information on the sensitizations detected. The medical report of a polysensitized patient in whom IgE reactivities to 70 of 112 allergen components were detected is given below by way of example.

The X-plain software can of course only deliver background information on the different allergens, and the results of the extensive sensitization test are not a substitute for an expert medical diagnosis. Therefore, all medical reports need to include a corresponding statement that the detection of IgE must always be evaluated in combination with the clinical medical history and that the computer-generated information is intended to assist the treating physician in making a clinical diagnosis and not to replace him/her.

In addition to the X-plain software developed by the manufacturer as an aid to interpretation, the "Allergenius" software-based expert system, which supports the

interpretation of ISAC data according to similar principles, was also introduced recently (Melioli et al. 2014). In addition to ISAC data, data from skin prick tests and individual sIgE determinations can also be entered in the Allergenius system and included in the computer-generated report. It can be assumed that expert systems such as X-plain or Allergenius will develop rapidly and further simplify the interpretation of complex sensitization profiles in the future (Matricardi et al. 2016).

Case Study: X-Plain Medical Report

Analysis of a Polysensitized Patient in Whom IgE Reactivities to 70 of 112 Allergen Components Were Detected

General Comments

The patient is polysensitized and exhibits IgE to cross-reactive as well as species-specific allergen components. IgE to peanut Ara h 2, peanut Ara h 6, peanut Ara h 9, hazelnut Cor a 8, Brazil nut Ber e 1, sesame seed Ses i 1, walnut Jug r 3, peach Pru p 3, soybean Gly m 6, wheat Tri a 14, hazelnut Cor a 9, peanut Ara h 3, soybean Gly m 5, and cashew nut Ana o 2 are associated with systemic allergic reactions. The higher the IgE level, the greater the likelihood of clinical symptoms.

Specific Components: Foods

IgE to specific allergen components of prawn, peanut, egg, Brazil nut, sesame seed, fish, soy, kiwi, hazelnut, wheat, milk, and cashew nut were detected (listed in descending order according to titer level):

- *Hen's egg*: A high level of IgE to Gal d 1 (ovomucoid) represents a risk marker for severe clinical reactions to both raw and cooked hen's egg and increases the risk of a persistent egg allergy. IgE to egg Gal d 2 and egg Gal d 3 are associated with reactions to raw or slightly heated hen's egg.
- *Milk*: IgE to milk Bos d 4 and milk Bos d 5 are associated with reactions to fresh milk.
- *Fish*: IgE to parvalbumin (cod Gad c 1), the major allergen from fish, can cross-react with parvalbumin from other fish species. Parvalbumin content varies considerably between fish species, which could explain differences in tolerance.
- *Crustaceans*: IgE to Pen m 2 can cause cross-reactions to crustaceans (e.g., crab, lobster) and insects (e.g., cockroach). IgE to Pen m 4 can cause cross-reactivity to related crustaceans (e.g., crab, lobster).
- *Nuts and legumes*: IgE to storage proteins (peanut Ara h 2, peanut Ara h 6, Brazil nut Ber e 1, sesame seed Ses i 1, soybean Gly m 6, hazelnut Cor a 9, peanut Ara h 3, soybean Gly m 5, and cashew nut Ana o 2) are associated with a risk for systemic clinical reactions. Many storage proteins are resistant to heat and digestion and are associated with allergic reactions to cooked and uncooked foods. Cross-reactions between soybean Gly m 6, hazelnut Cor a 9, and peanut Ara h 3 are possible. Cashew nut and pistachio are closely related. Walnut and pecan nut are closely related.

- *Wheat*: IgE to wheat Tri a aA_TI are associated with reactions to wheat-based foods. IgE to Tri a aA_TI are also associated with baker's asthma.
- *Kiwi*: IgE to Act d 1, a stable allergen from kiwi, are associated with severe reactions. Kiwi allergy sufferers that are not affected by an associated pollen allergy are at high risk for systemic reactions.

Specific Components: Aeroallergens

IgE to specific allergen components from grass pollen, birch, mite, dog, cat, olive, mouse, cockroach, pellitory, cypress, Japanese cedar, and plane were detected (listed in descending order according to titer level):

- *Pollen*: IgE to timothy grass components can cross-react with related proteins from other grass species. IgE to Bermuda grass Cyn d 1 and timothy grass Phl p 1 can cross-react. An elevated IgE level points to the primary sensitizing allergen. IgE to birch Bet v 1 (PR-10 proteins) can cross-react with related tree pollen and plant foods containing PR-10 proteins. The detection of IgE to Ole e 1, the major allergen from olive pollen, suggests sensitization to ash in areas with a high ash population. IgE to Ole e 9 from olive pollen is associated with severe respiratory symptoms (in areas with high olive pollen counts). IgE to plane Pla a 2 indicate genuine sensitization to plane pollen. IgE to Cry j 1 in areas where Japanese cedar does not occur naturally are a marker for sensitization to cypress. IgE to pellitory Par j 2 are an indication of species-specific sensitization with limited cross-reactivity to LTPs of other origin (e.g., from foods). IgE to Bermuda grass Cyn d 1, timothy grass Phl p 4, cypress Cup a 1, Japanese cedar Cry j 1, and plane Pla a 2 can be partially based on cross-reactivity to the CCD components of these native purified proteins.
- *Animal dander*: Fel d 1 is the major allergen from cat epithelium and triggers primary sensitization in cat allergy. IgE to dog Can f 2 and dog Can f 1 indicate genuine sensitization to dog. IgE to mouse Mus m 1 are associated with asthma and asthma morbidity. Mus m 1 is the major allergen from mouse epithelium.
- *Mites*: IgE to house dust mite Der f 2, house dust mite Der p 2, house dust mite Der f 1, and house dust mite Der p 1, the major allergen from house dust mite, were detected. Der p 1 and Der f 1 can cross-react. Der p 2 and Der f 2 can cross-react. IgE to Lep d 2 (storage mite) show less cross-reactivity with similar house dust mite proteins. IgE to mite Blo t 2 show limited cross-reactivity to Dermatophagoides; however, co-sensitization to both allergens occurs frequently. IgE to cockroaches is associated with asthma.

Specific Components: Insect Venom

IgE to bee venom Api m 1 is detected; further diagnostic testing is indicated in the case of clinically relevant insect venom allergy. All insect venom

components on the ISAC chip are CCD-free. This also applies to native bee venom component nApi m 4.

Cross-Reactive Aero- and Food Allergens

- *Serum albumin*: IgE to serum albumin can induce cross-reactivity between various animal species and cause allergic reactions following the consumption of meat and exposure to animal dander and epithelium. IgE to albumin can probably be attributed to sensitization to cow's milk, since milk contains bovine serum albumin.
- *Tropomyosin*: IgE to tropomyosins of house dust mite Der p 10, cockroach Bla g 7, prawn Pen m 1, and *Anisakis* Ani s 3 can explain allergic reactions to crustaceans (e.g., prawn, crab, escargot), mites, cockroaches, and parasites. Tropomyosin is heat-stable and can cause allergic reactions even when consumed in cooked form. Although tropomyosin is a major allergen in shrimps and other crustaceans, it is a minor allergen in mites.
- *Lipid transfer proteins (LTP)*: Even at low titers, IgEs to LTPs from foods (peanut Ara h 9, hazelnut Cor a 8, walnut Jug r 3, peach Pru p 3, and wheat Tri a 14) are risk markers for severe allergic reactions, particularly in Southern Europe. LTPs are predominantly found in the peel of fruits in the Rosaceae family as well as in nuts. These proteins are heat stable and can trigger allergic reactions even when consumed in cooked form.
- *PR-10 proteins*: In all likelihood, sensitization to PR-10 proteins was originally triggered by birch and predisposes affected individuals to allergic reactions (generally oral allergy syndrome) to fruits in the Rosaceae family as well as to hazelnuts, carrots, kiwi, and celery. Since PR-10 proteins are heat- and digestion-labile, they are generally tolerated in heated foods. A number of severe allergic reactions to Gly m 4, which occurred following the consumption of soy—often in combination with physical exertion and exposure—during the birch pollen season, have been reported

9.4.4 Special Features in Routine Use

Own experiences with the test system in routine diagnostics performed at a large outpatient allergy clinic have shown that positive sIgE values are rarely measured using ISAC 112 when total IgE concentrations are below 25 kU/l. Therefore, in our hands the test is now generally only performed when the total IgE concentration exceeds 25 kU/l (◉ Fig. 9.9).

Of the 112 allergens, six are glycosylated, i.e., have carbohydrate side chains that can bind IgE. These include walnut nJug r 2, Bermuda grass nCyn d 1, timothy

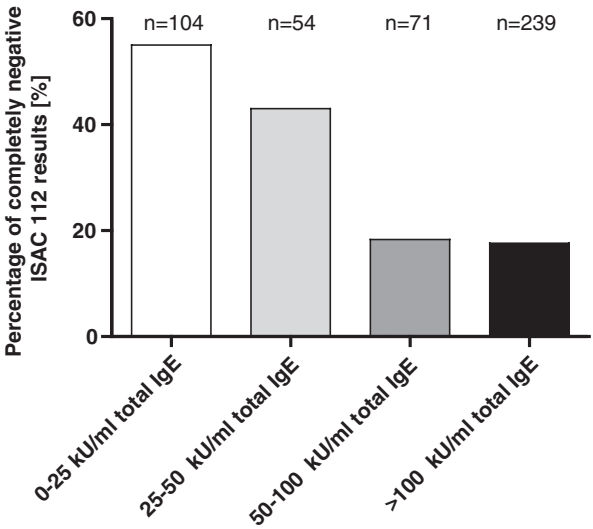

Fig. 9.9 The percentage of completely negative ISAC 112 results depending on total IgE

Table 9.6 Native glycosylated allergens bearing cross-reactive carbohydrate determinants (CCD) on the ISAC 112

Allergen source	Allergen	Protein family/biochemical name
Walnut	nJug r 2	Cupin
Bermuda grass	nCyn d 1	Grass group 1
Timothy grass	nPhl p 4	Unknown
Japanese cedar	nCry j 1	Pectate lyase
Arizona cypress	nCup a 1	Pectate lyase
Maple-leaved plane	nPla a 2	Polygalacturonase

grass nPhl p 4, Japanese cedar nCry j 1, Arizona cypress nCup a 1, and plane nPla a 2 (◉ Table 9.6). Since it is not possible to determine whether IgE to these six allergen components is directed to the protein or the carbohydrate side chain, the results need to be evaluated with caution and in the context of IgE reactivity to the CCD marker MUXF3 (van Ree et al. 2002).

9.5 Molecular Allergy Diagnostics Using Multiplex Assays in Research

9.5.1 New Insights Gained Using ISAC Technology

The small sample volumes required for multiplex assays are advantageous in the research environment, e.g., in the context of birth cohorts, since only small amounts of serum are normally available for analysis. These options made it possible to collect the following kind of data:

9.5.1.1 Diversity of Sensitization Profiles

By means of simultaneous determination of sIgE antibodies to numerous allergen molecules, patients' individual sensitization profiles can be generated with minimal effort. These profiles represent the IgE repertoire and pattern of sensitization at the molecular level and enable the great diversity of profiles in a population to be depicted. Tripodi et al. (2012) alone described 39 different profiles (sensitization patterns) in only 176 Italian, grass pollen-allergic children that were tested using eight *Phleum pratense* (timothy grass, Phl p) allergens: the spectrum extended from children who reacted to only one molecule, to children who produced antibodies to all eight allergens. A range of intermediate profiles exists between these two extremes.

9.5.1.2 Developing Sensitization Profiles

It could be shown using the ISAC method that sensitization profiles in children are simple to begin with and increase in complexity over time.

The sIgE response to the *Phleum pratense* (timothy grass) allergen molecules often develops from a simple monosensitization to a single allergen molecule into an oligomolecular sensitization, leading ultimately to a complex polymolecular pattern (Hatzler et al. 2012; Matricardi 2014). This development process usually begins with an IgE response to a initiator molecule, which, at later stages, initiates the development of antibodies to other allergen molecules. In the case of grass pollen allergy to timothy grass, this initiator molecule is usually Phl p 1, which turned out to be the protein most frequently recognized. As a result, young patients in the early stages of their sensitization often exhibit an sIgE response to only this protein. After months or years, IgE sensitizations to other timothy grass proteins can develop, commonly in a typical order: the initial sensitization to Phl p 1 is usually followed by positive reactions to Phl p 4 and Phl p 5; thereafter, IgE responses to Phl p 2, Phl p 6, and Phl p 11. Only in the clinical phase, long after all allergic symptoms had developed in these children, was it possible to detect IgE to Phl p 12 and Phl p 7–pollen panallergens with a low risk of sensitization. The time-dependent, consecutive development of allergen molecule-specific IgE sensitizations to an allergen source (grass pollen in this example) is described by the authors as "molecular spreading" (Hatzler et al. 2012).

Since the first sIgE responses to pollen are detectable years before the first symptoms occur, ISAC microarray analysis might be able to predict symptom onset on the basis of the individual sensitization profile. Indeed, approximately one-third of 3-year-old children sensitized to grass pollen develop grass pollen-associated seasonal rhinitis at the age of 12 years (Hatzler et al. 2012). Similar results were recently reported for the development of birch pollen-associated allergic rhinoconjunctivitis (Westman et al. 2015). Here again, IgE reactivity to various Bet v 1-homologous PR-10 proteins in early childhood seems to be a good predictor for the later development of a clinically manifest birch pollen allergy.

9.5.1.3 Prescribing Behavior in Allergen-Specific Immunotherapy (SIT)

Recommendations on SIT also take into consideration the efficacy of this therapy depending on how well it is adapted to the allergen sources to which the patient reacts (Zuberbier et al. 2010). SIT should be used in the case of clinical symptoms arising from IgE sensitizations to clearly definable allergen sources, including their primary major allergens, without taking cross-reactivity toward panallergens of questionable clinical relevance into consideration (Valenta 2002). The multiplex ISAC 112 system generates differentiated sensitization profiles, thus enabling "primary" genuine sensitizations to be distinguished from antibody reactions resulting from cross-reactivity. The advantage here is that it enables SIT to be individually tailored to each patient. Thus, current German language guidelines on SIT (Pfaar et al. 2014) recommend using specific single allergens in polysensitized pollen allergy patients—preferentially in singleplex rather than multiplex procedures—since generating complete sensitization profiles to answer the diagnostic questions would overshoot the target.

A multicenter Italian study (Stringari et al. 2014) has already investigated whether and how the results of molecular allergy diagnostics using singleplex assays influence physicians' prescription of SIT and decisions relating to the composition of allergen preparations for children with moderate to severe allergic rhinitis ($n=651$). This study revealed that more SIT preparations were prescribed following molecular diagnostics: in many patients originally classified as polysensitized on the basis of skin prick tests with pollen extracts, molecular diagnostics could identify clear sensitizations to particular major allergens, the allergen sources of which would then have come into consideration for SIT. The detection of IgE to primary marker allergens thus reestablishes the analytical specificity that was lost by using allergen extracts for diagnostic purposes due to pan-pollen sensitizations. In addition, it could be shown that, in approximately 33 % of cases, SIT would have been adjusted and performed with a different composition following molecular diagnostics.

9.5.2 The Use of Individually Tailored Allergen Chips in Research

In addition to the test systems approved for sIgE routine diagnostics (e.g., ImmunoCAP ISAC 112), protein microarrays can also be developed to address specific research interests. On the basis of ISAC technology, a significantly more extensive allergen chip was developed—e.g., for birth cohort-based investigations on the mechanisms of allergy development in different regions of Europe—on which a total of 176 allergen components are represented (Lupinek et al. 2014). In a similar manner, individually designed protein microarrays can be used as allergen chips in order to answer specific diagnostic questions. Thus, customized microarrays were able to detect sIgE to various chimeric isoforms of the Api m 10 major allergen in patients allergic to bee stings (van Vaerenbergh et al. 2015). The roles of sIgE to α-, β-, or γ-gliadin in wheat-dependent exercise-induced anaphylaxis were also

characterized using research microarrays (Hofmann et al. 2012), as was the relevance of the different single allergens for peanut allergy (Nicolaou et al. 2010).

A further application of array technology can be illustrated using the example of peanut allergy: rather than intact proteins, allergen peptides can also be coupled to the solid phase of the array as target structures. This type of peptide array permits the analysis of diverse linear IgE-binding sites (IgE epitopes) within an allergen (Shreffler et al. 2004) and their comparison with homologous sequences in other allergens (Rosenfeld et al. 2012).

The clear advantages of the multiplex assay for research purposes lie in the large number of detectable sensitizations, the individual composition of the allergen repertoire (personalized allergen chips), and the relatively small sample volumes required for the actual test. Particularly in the case of complex allergen sources and complicated clinical questions or in a polysensitized study population, high-definition molecular allergy diagnostics are beneficial, since the complete sensitization pattern obtained is a prerequisite for the successful interpretation of results in the context of the patient's clinical medical history.

9.6 Summary and Perspectives

The ISAC 112 microarray platform currently available enables the analysis of specific IgE to as many single allergens as possible in a single assay, using a small amount of serum (◉ Table 9.7). Strictly speaking, the assay represents 112 immunoassays, the corresponding allergen components of which are derived from natural or recombinant sources and have been individually evaluated for their suitability. This relates to allergen-dependent test parameters, such as LoD, linearity, precision,

Table 9.7 Advantages and disadvantages of test methods using the example of ImmunoCAP technology

Method	Advantages	Disadvantages
sIgE determination in ISAC multiplex assay	30 µl serum or plasma 112 allergen components No interference with high tIgE	Manual methods Less sensitive Higher coefficient of variation
sIgE determination in singleplex assay, e.g., ImmunoCAP	Automated Quantitative High test sensitivity Low coefficient of variation Well suited to monitoring/ follow-up	40 µl serum/plasma per analysis Low-affinity antibodies are also detected (virtually no clinical relevance)
Skin prick test (SPT)	High test sensitivity Simple and quick to perform	Manual One allergen per test Only extracts available

Adapted from Canonica et al. (2013)

effect of total IgE, IgE inhibition, matrix effects, and comparability with established methods for detecting specific IgE to define single allergens.

The analytical advantages of molecular diagnostics using single allergens also apply to multiplex analysis:

1. Increased test sensitivity (lower LoD) by using specific (e.g., allergens underrepresented or lacking in the allergen extract) single allergens
2. Increased analytical specificity (selectivity) for single allergens with defined clinical characteristics (e.g., risk association, disease association)
3. Defined single allergens (e.g., panallergens) as markers for cross-reactivity
4. Single allergens (e.g., species-specific marker allergens) as indicators of a primary, genuine IgE sensitization to the associated allergen source

The additional advantage of multiplex analysis is that it generates an extensive (ideally complete) IgE sensitization profile (complete allergen-specific IgE repertoire).

Since the reliability and accuracy of the current microarray test decrease significantly at sIgE concentrations below 1 kU/l, singleplex methods are—where possible—to be preferred over multiplex assays in the case of low serum total IgE (<25 kU/l) or only slightly increased sIgE values ($0.1 < \text{sIgE} < 1.0$ kU_A/l).

A number of important allergen components, particularly in the area of food allergens (e.g., additional storage proteins; missing, potentially important pollen allergens; mold allergens; animal allergens) are lacking. Other allergen components currently featured on the allergen chip would be better dispensed with, since they lead more to confusion than to clarification. These include insect venom allergens, since analysis of specific IgE to these allergens is only indicated on the basis of clear signs of an anaphylactic reaction to insect stings in the patient's medical history, and not as a screening test. Due to the high prevalence of insect venom sensitization in approximately 25 % of the population, nonspecific screening would generate an abundance of clinically irrelevant results and serve to unsettle patients and their physicians. On the basis of the appropriate indication, sensitization to single insect venom allergens can be detected using singleplex methods. Alternatively, specific multiplex analysis with all available insect venom allergens—a test currently under development and known as the insect venom allergen chip—would be useful. In this regard, it is conceivable that a range of microarray formats will be available in the future, which, depending on the clinical question, will cover different allergen spectra, such as food allergies, inhalant allergies, insect venom allergies, and medication allergies. In light of the fact that there are probably over 3000 single allergens, it can be expected that the rapid developments in miniaturization and automation will fuel many more innovations in the field of multiplex allergy diagnostics.

Conclusions

The ISAC 112 microarray platform currently available represents an important step in the further development of in vitro allergy diagnostics in that it enables

the analysis of specific IgE to as many single allergens as possible in a single assay, using a small amount of serum. The advantages of molecular allergy diagnostics (greater test sensitivity, increased analytical specificity, and the ability to identify risk, primary, and cross-sensitizations) are broadened by the comprehensive generation of virtually complete sensitization profiles.

Positive IgE microarray results indicate sIgE sensitizations to the relevant single allergens—sensitizations that are only clinically relevant in the presence of corresponding symptoms following exposure to the associated allergen source. Clinical relevance needs to be investigated for each allergen source or single allergen separately, possibly by means of a targeted follow-up patient history or, where possible, by means of challenge testing with the relevant allergen source. Conversely, IgE sensitizations detected on the microchip in the absence of clinical information on physical symptoms, allergic reactions, or individual diseases in the affected individual are of limited value: neither the level of sIgE nor the extent or pattern of IgE sensitizations to single allergens reveals anything about their potential clinical relevance. The IgE sensitizations detected can only be conclusively interpreted in combination with knowledge of the clinical symptoms. This remains the task of the physician and cannot be substituted even by detailed information on sIgE to all conceivable single allergens.

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