

The Allergen: Sources, Extracts, and Molecules for Diagnosis of Allergic Disease



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Overall Purpose/Goal: To provide excellent reviews on key aspects of allergic disease to those who research, treat, or manage allergic disease.

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Learning objectives:

1. To recognize the differences between using standardized or non-standardized extracts for diagnostics.
2. To recognize clinical implications of sensitization to specific allergenic proteins (components).

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Allergenic source materials include pollen, molds, animal dander, and insects; food allergens from nuts, grains, and animals; venoms; and salivary proteins from insects and ticks. Clinical diagnostic tests have used heterogeneous extracts from allergen source materials for skin prick tests (SPTs). *In vitro* laboratory methods using immunoassays or microarrays can detect serum IgE directed against allergenic proteins where clinical testing may not be suitable. Clinicians rely primarily on

licensed commercial extracts of allergens for SPTs. Manufacturers and regulatory agencies have standardized selected extracts for identity, composition, and potency. Allergen sources contain multiple proteins. The IgE antibody responses to these proteins vary between allergic subjects as does the quantity of specific IgE. Component-resolved molecular diagnostics can be used to improve the specificity of allergy testing and resolve clinical cross-reactivities that may affect treatment outcomes.

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Abbreviations used

cGMP- Current Good Manufacturing Practice

EDQM- The European Directorate for Quality of Medicines and Healthcare

FDA- US Food and Drug Administration

IUIS- International Union of Immunological Societies

mAb- Monoclonal antibodies

MS- Mass spectrometry

NMR- Nuclear magnetic resonance

PNU- Protein nitrogen unit

RAST- Radioallergosorbent test

SPT- Skin prick test

WHO- World Health Organization

This clinical commentary will review methods for the production, evaluation, and standardization of allergen extracts from the perspective of diagnostic testing that may be useful for allergists in practice. © 2020 American Academy of Allergy, Asthma & Immunology (J Allergy Clin Immunol Pract 2020;8:2506-14)

Key words: Allergens; Standardization; Molecular diagnostics; Allergy testing; Component-resolved diagnostics; IgE responses

There has been marked evolution in the manufacture and use of allergenic materials as *in vivo* diagnostic reagents since 1873, when Charles Blackley determined that grass pollen would elicit hay fever symptoms when applied to his nasal mucosa or conjunctivae.¹ In 1906, von Pirquet coined the term “allergy” to describe the immediate reactions to antigen in the skin.¹ In the 1940s, aqueous extracts of allergenic materials such as short ragweed pollen (*Ambrosia artemisiifolia*) were used in skin testing to diagnose hay fever caused by inhalation of pollen.² The discovery of IgE by the Ishizaka's in 1967 as the mediator of immediate hypersensitivity responses seen in skin prick tests (SPTs) allowed *in vitro* tests for measuring IgE antibodies in serum to be developed.³ As allergen extracts became available for a greater number of plant, insect, fungal, and animal species and allergen skin testing grew in popularity among allergists, the purity of the test materials was questioned in relation to accuracy of diagnosis.⁴ This prompted research to identify the relevant allergens from many different sources with the aim of defining the most important, clinically relevant, molecular entities. One outcome of this research was that in many cases selected allergens that bound to serum IgE in >50% of allergic individuals were purified. Specific IgE for these molecules could be used as diagnostic markers for sensitization as an indicator of clinical allergy. A systematic nomenclature was developed for the identification and naming of allergens, which is maintained by a subcommittee of the International Union of Immunological Societies with the World Health Organization (WHO/IUIS) using a database that is publicly available (www.allergen.org). The committee reviews candidate allergenic proteins that have human serum IgE binding data and assigns the correct nomenclature.⁵ However, names for allergens are often assigned before publication, and a few of the 977 named allergens in the database are not supported by published proof of allergy. The WHO/IUIS database is the primary source for allergen nomenclature. A second allergen database that provides postpublication reviews of data and sequence comparisons is the www.allergenonline.org database of

the Food Allergy Research and Resource Program at the University of Nebraska.⁶ It connects the taxonomy of the source and the proteins that are identified as allergens.

This clinical commentary will review biological source materials and established manufacturing methods used to prepare allergenic products for use by allergists in clinical practice. The relative merits of standardized and nonstandardized extracts are compared together with factors that influence allergenic potency. We overview purified allergen proteins used in component-resolved or molecular diagnostics as new tools for differential allergy diagnosis. Technological advances and quality standards for allergen molecules are reviewed, together with representative examples of the clinical relevance of IgE molecular diagnostics.

ALLERGENS IN SPECIFIC EXTRACTS

Over more than 40 years, researchers have identified and characterized hundreds of proteins that bind human IgE and, in some cases, cause the degranulation of basophils or skin mast cells. However, the amount and potency of the various allergenic proteins in extracts have not been documented for most allergen extracts. In principle, the potency of a diagnostic allergen extract should reflect its ability to induce degranulation of skin mast cells in allergic individuals. Because allergen extracts are heterogeneous complexes and the allergens recognized by affected individuals may vary, the relevant allergens that need to be measured in an extract are not simple. For those few allergen extracts that are approved as standardized by the US Food and Drug Administration (FDA), relevant allergens or allergen activities are being measured.

Short ragweed pollen and perennial ryegrass pollen extracts were standardized by intradermal skin tests using intradermal injections of extracts in subjects with allergy to these pollen species. The technique was developed by scientists at the FDA and Johns Hopkins University.⁷ Allergen extracts in buffered saline with 50% glycerol and at 4°C to 8 °C maintain their potencies for 3 years.⁸ The first purified protein fraction of a ubiquitous outdoor mold *Alternaria alternata*, previously called *Alternaria tenuis*, was quantitated by a radioallergosorbent test (RAST) inhibition assay using sera from 16 allergic subjects having rhinitis and/or asthma coincidental with the *Alternaria* season.⁹ The serum donors were qualified by a positive skin test, IgE antibody measurement, bronchiolar inhalation tests, and leukocyte histamine release analyses. The RAST inhibition assay has become a model assay for comparing the potency of allergens in the United States. Major and minor allergens of chicken egg white have been determined by similar methods that showed strong reactivity for ovalbumin and ovomucoid and much lower reactivity for lysozyme and conalbumin.¹⁰

NONSTANDARDIZED EXTRACTS

Most extracts used for allergy diagnosis and immunotherapy in the United States are not standardized and do not have potency assignments similar to standardized products. Extracts from common grass pollens, weed pollens, and tree pollens, although nonstandardized, have been licensed and used successfully in skin tests to identify allergic sensitization. Likewise, food extracts have not been standardized. However, cashew nut extracts have been characterized for potency. In clinically defined study subjects, positive SPTs with characterized cashew extract with confirmatory specific IgE determinations were predictive of positive food challenges.¹¹ Tree nut extraction must be

optimized for individual species as protein solubility varies especially for walnut and possibly other nuts.

INFLUENCE OF CLIMATE CHANGE ON ALLERGENS USE IN DIAGNOSIS

Changing weather patterns involving climate change can modify the expression of some allergenic proteins in pollen. Increasing carbon dioxide and nitric oxide in the atmosphere can increase the expression and abundance of some allergens including Amb a 1 by 1.5% or more and some other allergens in ragweed pollen in controlled studies as well as increased global distribution of the plant itself from North America to Europe and much of Asia.¹²⁻¹⁴ Changing weather and NO in the atmosphere may play important roles in the geographic prevalence of environmental allergies to certain pollens and fungi. Correct diagnosis requires the availability of well-characterized extracts, whether standardized or nonstandardized, and an assessment of the geographical distribution of the allergen species relative to the patient's domicile.¹⁵ The geographical location and movement of people exposes them to different aeroallergens. Thus, the allergist should investigate the history of their patients. For instance, people who live or once lived in areas where birch pollen or alder pollen are common can produce IgE antibodies that can bind to the homologous Gly m 4 protein in soybean, making the diagnosis of soy allergies problematic.¹⁶

How allergen extracts are made

The manufacture of allergen extracts has 3 essential components: source material selection and collection, extraction, and filtration and storage.

Source material selection and collection. Because allergen extracts are essentially unselective aqueous extraction products, the choice and handling of the allergen source material is *the single most important step in manufacturing*. Source material selection and handling is especially critical for US allergen extracts that are nonstandardized (see below). Important criteria to consider in the selection of source materials include:

- **Speciation:** For some materials (animals, pollens, mites, molds, insects, most foods) speciation is straightforward. For others (wild-caught insects, store-bought fish, and seafood) speciation can be challenging and manufacturers will need appropriate expertise to do so with confidence.
- **Part selection:** Species identification is not the only feature of a source material that requires identification and control. An organism has many distinct anatomical parts. For some allergen extracts (pollens, animal danders and pelts, Hymenoptera venoms), those parts are named with specificity. However, other extracts are named without part designation, even though allergen content may be significantly affected by parts included in the extraction. Examples include dust mites and cockroaches, where feces and, for cockroaches, egg casings are enriched for allergens not otherwise present in the whole bodies. Product consistency can only be assured if proportional part content is maintained.
- **Environmental factors:** Other factors that warrant attention, if not control, are time and weather (pollen allergen content may vary by season) and other environmental factors (mold content varies by growth conditions and life cycle stage).

- **Cleaning and initial preparation:** Depending on the species, most source materials need to be washed and defatted before further processing. Pollens and animal danders are sieved and washed to reduce airborne contaminants; cultured mites are sieved and washed to remove culture media; mold cultures may be washed to remove culture media unless elemental media are used.¹⁷

Defatting produces an allergen source material that is largely decolorized and easier to store, apportion, weigh, and extract. It has been assumed, erroneously, that the removed lipid-soluble components are largely nonallergenic, although even older protocols recognized that the fat-soluble residue could be retrieved and used for patch testing.¹⁸ Once selected, cleaned, and tested, manufacturers need to store source materials properly to preserve relevant allergen content, with attention to containers, temperature, and humidity.

Most allergenic proteins are rapidly soluble in water and well represented in aqueous extracts. However, for both standardized and nonstandardized allergenic extracts, the possibility exists that certain important allergen molecules will be missing from the extracted material. For example, the latex allergen Hev b 5 was missing from early glove extracts and Pru ar 5 was missing from some aqueous peach and apricot extracts, even though molecular diagnostics have shown these to be important allergens.¹⁹

Extraction and filtration. Allergen extracts are prepared from source materials by aqueous extraction in one of several buffered saline solutions. Extraction is performed at 4°C to 8°C for 24 to 72 hours under agitation. The extract is then separated from the extracted source material by serial centrifugation and/or filtration; a "settling" step is sometimes needed to further clarify the extract. Aseptic filtration through a 0.2-μm filter precedes aseptic vial filling, typically into a sterile bulk container. Glycerol and phenol, which function as preservatives and stabilizers, may be added to the extraction buffer before extraction or after; less often, human albumin (0.03%) is added as a stabilizer.²⁰

Storage. Standardized allergen extracts undergo stability testing to define the optimal storage conditions and duration. Because the unitage for nonstandardized allergen extracts cannot support scientifically valid stability evaluations, the expiry for these products was established in the regulations (formerly 21 CFR 610.53) and incorporated into the respective biological license applications at 3 to 6 years from the date of manufacturing for glycerinated products.

How the FDA regulates allergen extracts

Over 40 years, 2 aspects of the FDA regulatory program have had a significant impact on allergen manufacturers and have enhanced the safety of allergen extracts marketed to the American public.²¹ The first feature is the enforcement of *current Good Manufacturing Practice* (cGMP) standards (21 CFR 210, 211, and 680) on the manufacture of allergen products. cGMP standards are broad and comprehensive, and address organization and personnel, buildings and facilities, equipment, control of components and drug product containers and closures, production and process controls, holding and distribution, quality control, laboratory controls, and records and reports.

The second feature is the standardization of allergenic extracts. *Standardization* is the process by which a reference extract is selected, and methods and procedures are developed to

quantitatively compare a test extract with a reference extract to establish equivalence.²¹⁻²⁴ Allergen standardization became a reality in the United States in the late 1970s when the first allergen extracts were produced to meet FDA criteria for potency and composition. Standardization ensures that the assayed property of the extract is clinically relevant for defining potency and safety and that test methods for estimating the clinically relevant property are accurate and precise.²⁵ The FDA standardized allergenic extracts for *in vivo* human use are assessed for biological potency by quantitative intradermal skin testing, together with *in vitro* IgE testing and biochemical evaluation of the extract composition. Biological testing is important because the extracts are for *in vivo* use. Extracts that have been validated by biological testing are often incorporated into *in vitro* tests. However, *in vitro* IgE tests do not require GMP manufactured allergens and are validated for performance parameters using clinical laboratory practices to assess allergic sensitization.^{23,24}

Unitage for the standardized allergen extracts is assigned based on the nature of the allergens and the immunodominant immune response. The *specific allergen content* of certain allergen extracts is the basis for unitage for 2 standardized allergenic extracts, short ragweed pollen and cat hair/pelt, for which the immunodominant allergens (Amb a 1 and Fel d 1, respectively) have been identified and defined. For those standardized extracts where no immunodominant allergen has been identified (mites and grass pollens), overall IgE binding forms the basis for unitage. Finally, the Hymenoptera venoms are labeled based on mass unitage of venom or venom protein.

In contrast, nonstandardized allergen extracts have no US standard of potency and are manufactured and labeled based on *protein nitrogen unit* (PNU) or on the weight of the source material extracted with a given volume of extracting fluid (w/v).²⁶ The PNU or w/v units for these extracts have no direct correlation to biological potency, and, unlike standardized allergen extracts, no formal assessments of nonstandardized allergen extract efficacy have been performed. The level of detailed knowledge needed across-allergic populations and variation in allergic responses in individuals and populations provides enormous challenges for selecting and verifying standards. In many cases, multiple allergens have been identified within 1 source material with IgE binding from some population of allergic subjects. The variation of the concentration of allergens has been noticed in various similar sources that may represent genomic differences or environmental differences. Standardization based on a fixed concentration of 1 or 2 proteins under the reality of such variation would likely not benefit the majority of the allergic population. The value of the nonstandardized extracts for diagnosis depends to a great degree on the source material identity and quality, extraction efficiency, and preservation of proteins as controlled by the individual manufacturer.²⁷ The responsibility of the FDA then is to inspect the facilities and procedures and ensure that the extracts are safe and correctly identified as to product labels.

Efficacy review activities (1972+) and conclusions

When regulation of allergen extracts transferred from the National Institutes of Health to the FDA in 1972, the Agency initiated a broad review of the efficacy and safety of these important products.²⁸ As previously noted, “the transfer of allergenics to the FDA had 2 major implications for manufacturers and their products. First, allergen extracts were now under the

jurisdiction of a regulatory agency with an unambiguous and mature compliance-based apparatus. Second, allergenics were now subject to the 1962 Kefauver-Harris amendments, which require manufacturers to submit evidence of effectiveness and not just safety before marketing approval.” That review was achieved in multiple stages, involving external experts and internal scientific and medical staff.

Notable features of the review, in all stages, included an exclusive dependence on published literature and an acceptance of well-described case reports and certain cross-reactivity data as supportive of effectiveness. Applying these standards, only half of reviewed licensed allergen extracts had literature that supported effectiveness.

IMPLICATIONS FOR PRACTITIONERS AND PRACTICE GUIDELINES^{29,30}

- Allergen extracts are potent and stable: Standardized allergen extract potency is based on the measurement of biologically relevant allergens or immune response (Table I). For the nonstandardized allergen extracts this cannot be done—assurance of potency relies on consistency of the source materials and manufacture processes. FDA regulations and inspections increase the likelihood of maintaining this consistency, but there are limitations imposed by this reliance, notably the inability to make lot-to-lot comparisons and assess stability over time.
- Standardized extracts are more clinically reliable than nonstandardized extracts, but nonstandardized extracts may be useful nonetheless: based on the FDA’s efficacy review of nonstandardized allergen extracts, only half of these products had literature that supported their effectiveness.²⁸ These are licensed products, but their licenses were issued long before the current clinical standards were in place. As noted in an Advisory Committee discussion at the time (Allergenic Products Advisory Committee meeting, 2011),³⁴ FDA staff relied on published data alone to support their conclusions. Clinicians and researchers alike should be aware of the limitations of these products and take this into account when using them.
- Clinical utility of fruit/vegetable extracts: All food allergen extracts in the United States are nonstandardized. As with all nonstandardized allergen extracts, no valid potency measure exists for these extracts. In practice guidelines,²⁹ based on a study of individuals with oral allergy syndrome,³⁵ it is suggested that skin testing with fresh fruits/vegetables may be necessary to diagnose these allergies, even though the risk of a generalized reaction to fresh food testing may be higher in young children.
- Clinical utility of dog extracts: Nonstandardized dog allergen extracts exist in the United States in 2 formulations, conventional and “acetone-precipitated.” As with all nonstandardized allergen extracts, no valid potency measure exists for these dog extracts. In spite of this, authoritative literature reports³⁶ and practice parameters³⁰ suggest that the use of the acetone-precipitated product will yield better clinical results associated with their higher Can f 1 levels. These recommendations are not based on clinical studies, and a recent report suggests that the immune response to the dog is more complex than previously thought.³⁷

In general, the standardized allergen extracts were licensed more recently, and under more stringent regulatory

TABLE I. Standardized and licensed allergen extracts in the United States

Allergen extract		Current lot release tests	Labeled unitage
Dust mite (<i>Dermatophagoides farinae</i>)		Competition ELISA	AU/mL (equivalent to BAU/mL)
Dust mite (<i>Dermatophagoides pteronyssinus</i>)		Protein*	
Cat pelt (<i>Felis domesticus</i>)		Fel d 1 (RID)	BAU/mL
Cat hair (<i>Felis domesticus</i>)		IEF	5-9.9 Fel d 1 U/mL = 5000
		Protein	BAU/mL
			10-19.9 Fel d 1
			U/mL = 10,000 BAU/mL
Bermuda grass pollen (<i>Cynodon dactylon</i>)		Competition ELISA	BAU/mL
Red top grass pollen (<i>Agrostis alba</i>)		IEF	
June (Kentucky blue) grass pollen (<i>Poa pratensis</i>)		Protein*	
Perennial ryegrass pollen (<i>Lolium perenne</i>)			
Orchard grass pollen (<i>Dactylis glomerata</i>)			
Timothy grass pollen (<i>Phleum pratense</i>)			
Meadow fescue grass pollen (<i>Festuca elatior</i>)			
Sweet vernal grass pollen (<i>Anthoxanthum odoratum</i>)			
Short ragweed pollen (<i>Ambrosia artemisiifolia</i>)		Amb a 1 (RID)	Amb a 1 units
Yellow hornet (<i>Vespa</i> species)		Hyaluronidase and	µg protein
Wasp (<i>Polistes</i> species)		phospholipase activity	
Honey bee (<i>Apis mellifera</i>)			
White faced hornet (<i>Vespa</i> species)			
Yellow jacket (<i>Vespula</i> species)			
Mixed vespid (<i>Vespa</i> + <i>Vespula</i> species)			

Product		Manufacturer	Unit	Basis
Parenteral	Nonstandardized	Several companies	PNU/mL w/v	Measured protein or mass/volume
Sublingual tablets	Short ragweed pollen	ALK	Amb a 1 units	Based on skin prick test size. 100 IR has same geometrical mean diameter wheal size as codeine phosphate control ^{31,32}
	Timothy grass pollen	ALK	BAU	
	Sweet vernal, orchard, perennial rye, timothy, and Kentucky blue grass pollens	Stallergenes/Greer	IR	
	Mites	ALK	SQ-HDM	
				Based on biologic potency, major allergen content and complexity of the allergen extract ³³

AU, Allergy units; BAU, bioequivalent allergy units; ELISA, enzyme-linked immunosorbent assay; IEF, isoelectric focusing; RID, radial immunodiffusion.

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*Test for informational purposes only.

requirements, than the nonstandardized extracts.³⁸ Other recent allergen product approvals (Prescribing information—GRAS-TEK grass tablet; PALFORZIA, peanut powder; RAGWITEK, ragweed extract inclusion for dysphonia; ORALAIR, 5 grass extract; ODACTRA, house dust mite extract)^{31,33,39-41} were conducted under a modern, rigorous review program that requires affirmative evidence of clinical effectiveness and safety in addition to consistent safe manufacture under cGMP, and labeling that expresses meaningful biological potency.

A challenge for clinicians, manufacturers, and regulators is the fact that patients are exposed to a variety of allergenic sources, and the immune responses in a population to these materials may be heterogeneous. For example, the major peanut allergens are Ara h 1, Ara h 2, Ara h 3, and Ara h 6. Yet in northern Europe where people are often sensitized to birch pollen, the Bet v 1 homolog Ara h 8 can be an important allergen for a small number of patients.⁴² People in Spain and Italy may be sensitized to lipid transfer proteins and be sensitive to Ara h 9.⁴³ If peanut extract is standardized by the concentrations Ara h 1, 2, 3, or 6, levels of Ara h 8 and Ara h 9 will not be adequately controlled.

ALLERGEN MOLECULES—THE ESSENTIAL COMPONENTS OF MOLECULAR DIAGNOSTICS

Allergens are among the most well-defined groups of molecules in biomedical research in terms of their structure, biologic effects, and function. The WHO/IUIS Allergen Nomenclature website lists 979 allergens included in the systematic nomenclature.⁴⁴ More than 500 allergens have been sequenced and expressed, and, remarkably, more than 100 structures of allergens and allergen antibody complexes are listed in the Protein Database (Table II).^{5,45} Allergens belong to a diverse group of 180+ protein families and include, among others, enzymes, ligand binding proteins, seed storage, and pathogenesis-related proteins.^{3,4,46}

The tremendous progress in allergen proteomics has improved the manufacture of conventional diagnostic and therapeutic allergenic products, greatly facilitated allergen standardization worldwide, and has underpinned the development of component-resolved or molecular diagnostics, ushering in a new era of personalized medicine in allergy.²⁴

Several technological advances have fueled this progress:

TABLE II. Allergen molecules—at a glance

- 979 allergen molecules in the systematic WHO/IUIS Allergen Nomenclature Database (www.allergen.org)
- >500 allergens cloned, sequenced, and expressed as recombinant proteins
- ~108 three-dimensional allergen structures in the Protein Database (PDB), www.rcsb.org. An additional 20+ structures of allergen-antibody complexes are in the PDB
- Occur in approximately 180 families in the Protein Family database (Pfam); have diverse structures and biologic functions (www.sanger.ac.uk/Software/Pfam)
- Can be manufactured at 95% to 99% purity and validated using monoclonal antibodies, IgE antibodies, and mass spectrometry

IUIS, International Union of Immunological Societies; WHO, World Health Organization.

- *Monoclonal immunoassays* are used for precise quantification of major allergens in allergenic products used in clinical practice. Allergen measurement is required by regulatory authorities in Europe and has enabled manufacturers to improve the lot-to-lot consistency and reproducibility of their skin test and therapeutic extract products.^{26,47} In the United States, measurements of Amb a 1 and Fel d 1 are required by the FDA for potency assessment of short ragweed pollen and cat allergen extracts. Thus, cat extracts with a potency of 10,000 BAU/mL contain 10-19.9 FDA units/mL Fel d 1 and can be used to determine doses of allergen administered over the course of immunotherapy.⁴⁸ The European Directorate for Quality of Medicines and Healthcare (EDQM), together with the Paul Ehrlich Institute (Langen, Germany) and researchers in academia and industry, has developed a certified enzyme-linked immunosorbent assay for birch pollen allergen, Bet v 1, as part of the BSP 090 allergen standardization project.⁴⁹ This assay is expected to be included in the European Pharmacopoeia in 2020. In both cases, the outcome for allergists is greater comparability of cat and birch pollen extracts between manufacturers and more reliable and consistent dosing to increase the safety and efficacy of immunotherapy.
- *Recombinant allergens* are manufactured in high-level expression vectors, including *Escherichia coli* and *Pichia pastoris*. Recombinant or purified natural allergens are the molecular targets for IgE antibodies and are being used to develop the next generation of *in vitro* tests for allergy diagnosis. Purified allergens are being developed as empirical standards for immunoassays and mass spectrometry (MS). As part of the BSP 090 project, the EDQM consortium has produced and validated recombinant allergen standards for Bet v 1 and Phl p 5 that are part of the European Pharmacopoeia.⁵⁰
- *X-ray crystallography* and nuclear magnetic resonance (NMR) analyses of the 3-dimensional structure of allergens.⁵¹ Advances in the computation of X-ray diffractions patterns (eg, using HKL 3000 software) allow crystal structures to be resolved within days of obtaining allergen or antibody crystals.⁵² Structures can be resolved within months rather than years. Structural studies allow the precise location of amino acids involved as isoforms, variants, IgE, or monoclonal antibody binding and catalytic sites to be determined on or within the allergen molecule. They provide the molecular basis for allergenic cross-reactivity and allow further investigation of clinical significance.

- *MS* is coming of age and being used for compositional analyses of allergenic extracts and to assess the purity of purified allergens for diagnostic use.⁵³⁻⁵⁶ The latest generation of “benchtop” Orbitrap instruments with multiplexing capabilities provide sophisticated analyses of protein identification, post-translational modifications, relative protein abundance, and absolute protein quantification.^{56,57} MS has multiple applications in allergen proteomics. Natural extracts, for example, cockroach, can be analyzed to determine the repertoire and concentration of allergens present in the sample using multiple reaction monitoring MS.⁵⁴ Milk caseins (Bos d 8) can be analyzed to determine the relative abundance of α -, β -, and κ -caseins. Peanut flour products for use in preclinical and clinical trials as early intervention therapeutics are being formulated based on Ara h 1, Ara h 2, Ara h 3, and Ara h 6 compositions. MS is particularly useful when immunoassays are not available. Another key advantage of MS is the monitoring of allergenic products or foods that have been chemically modified or denatured (eg, allergoids) or processed by heat or other food processing methods. Finally, MS is a critical tool for analyzing the purity of allergens used in molecular diagnostics. For natural allergens, trace contamination with other allergens can be determined, and for recombinant allergens, contamination with host cell proteins can be assessed.⁵³ Accurate and absolute quantification of allergen proteins use in molecular diagnostics or as allergen reference materials can be achieved using isotope-labeled peptides in MS.^{54,58,59}

Together, monoclonal immunoassay, recombinant allergens, X-ray crystallography/NMR, and MS have transformed the field of allergen proteomics, which is essential for allergen-specific molecular diagnostics and new therapeutic strategies. There have been more than 15 clinical trials of allergen immunotherapy recombinant allergens.⁶⁰ Most of these trials have used Bet v 1 for the treatment of birch pollen allergy or several recombinants for grass pollen allergy. In most cases, measurements of these major allergens, together with cGMP allergen manufacturing and purity assessments, similar to those described here, have been used to validate recombinant allergens for therapeutic use.

PURIFIED ALLERGENS FOR MOLECULAR DIAGNOSIS OF ALLERGIC DISEASES

There would be several significant advantages of the use of purified, recombinant allergens for the skin test diagnosis of allergic diseases including manufacturing supply, product consistency, and, in principle, more accurate diagnosis and better immunotherapeutic options.⁶¹ Although regulatory pathways for the approval of such *in vivo* products are available, none are on the market anywhere at this time.

The situation regarding *in vitro* testing, however, is more promising. Assessments of IgE-mediated sensitization using purified allergens in *in vitro* tests have several advantages compared with use of allergen extracts: (i) monitoring of IgE responses to individual allergens from multiple sources; (ii) greater diagnostic precision and unambiguous determination of cross-reactivity; (iii) improved diagnostic efficacy and risk assessment; and (iv) association of IgE responses to specific allergens as predictors of disease progression and severity.^{46,62} Sensitization to birch pollen is a classic example of molecular diagnostics in action. Patients sensitized to Bet v 2 (profilin) frequently develop oral allergy

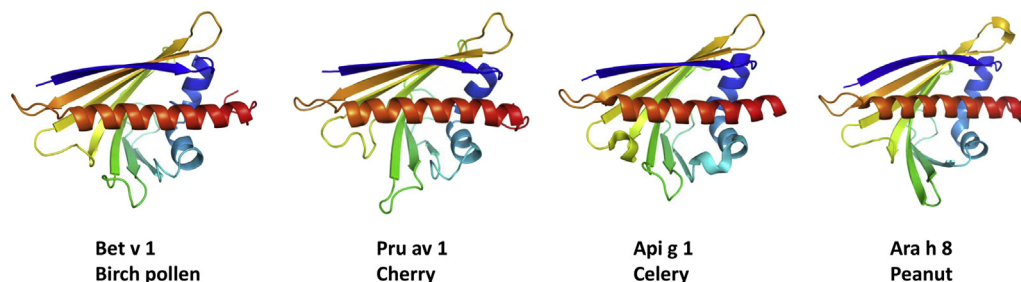


FIGURE 1. Structural homology between birch pollen and food allergens that are pathogenesis-related group 10 proteins. Patients who are primarily sensitized to Bet v 1 may also show cross-sensitization to cherry, celery, or peanut and may have oral allergy syndrome. In Central Europe, where consumption of celeriac is common, Api g 1 is the primary cause of sensitization and food allergy symptoms.

TABLE III. Allergen molecules as predictors of disease severity

Allergen(s)	Disease severity
Der p 1, Der f 2, Der p 23	Asthma
Bet v 1	Allergic rhinitis, asthma
Fel d 1	Allergic rhinitis, asthma
Phl p 1, Phl p 7	Allergic rhinitis, asthma
Phl p 12	Oral allergy syndrome
Ara h 2	Anaphylaxis, severe food allergy

syndrome as a result of cross-reactivity with soft fruits and vegetables, which also contain homologous cross-reactive profilin allergens. Patients sensitized to Bet v 1 may also have IgE antibodies to peanut allergen, Ara h 8, and show positive skin test responses to peanut. These reactions are caused by the structural and antigenic homology between Bet v 1 and similar pathogenesis-related proteins found in cherry (Pru av 1), celery (Api g 1), and peanut (Ara h 8) (Figure 1). However, patients with IgE to Ara h 8 are at low risk of developing severe reactions to peanut and, in most cases, can consume peanuts as part of their diet without adverse effects. In several cases, the measurement of IgE to specific allergens (eg, Fel d 1, Can f 1, Ara h 2, and Phl p 1) is a better predictor of clinical sensitivity than measuring IgE to allergen extracts.⁴⁶ Evidence is emerging that sensitization to 1 or more “initiator” molecules in early life predicts progression to allergic rhinitis, asthma, or food allergy later in life. In the German Multicenter Allergy Study cohort, sensitization to Der p 1 and Der p 23 in the first 5 years of life was associated with asthma at age 12, whereas in the Manchester Asthma and Allergy Study cohort, Der f 2 was also predictive. These studies also showed that “molecular spreading” of IgE responses to a larger number of allergens was associated with greater disease severity. Similar disease progressions have been associated with IgE responses to other major allergens (Table III).⁴⁶

Multiplex technologies and “lab-on-a-chip” nanotechnology are used in microarrays for allergy diagnostics.^{63,64} The earliest and most widely used microarray has 112 allergens on a glass slide and can be used to test 4 sera.⁶⁵ Recently, under the European Union–funded project “Mechanisms for the Development of Allergies (MeDALL),” an array of 170 allergens has been developed (<http://medall-fp7.eu/>).⁶⁶ Most allergy diagnostics manufacturers have incorporated molecular diagnostics into their IgE test platforms, and several biotech start-ups are entering the

field to develop better, faster, patient-centered diagnostic technologies. The broad spectrum of allergens for which IgE determinations are being made prompted the European Academy of Allergy and Clinical Immunology to publish a 250-page molecular allergology user guide to aid allergists and other clinicians in result interpretation and clinical significance.³² The European Academy of Allergy and Clinical Immunology user guide includes recommendations for the diagnostic workup of allergic patients using molecular diagnostics, together with the benefits and limitations of this approach using single or multiple allergen components.³² This issue of the journal also contains a detailed review of methods used for sIgE molecular diagnostic testing.⁶⁷ One downside, especially for large static arrays of 100+ allergens, is that much of the IgE data generated may not be relevant to an individual patient. Efficient data processing could alleviate this problem, together with the development of flexible, focused arrays for allergen groups—foods, indoor allergens, pollens, venoms.⁶⁷ Yet, appropriate diagnosis always requires the clinician to consider the specific history of the patient and information related to exposure and symptoms because specific IgE alone only suggests plausible causes of allergy.³²

Manufacturers face several hurdles and challenges in developing new diagnostic devices.⁵⁶ Producing 100+ purified natural or recombinant allergens with validated purity, immunoreactivity, and IgE binding activity requires significant investment in infrastructure, process development, and scientific expertise. Some recombinant allergens are difficult to express (eg, pectate lyase allergens from weed and cedar pollens).⁶⁸ However, the most pressing challenge faced by manufacturers is sourcing sera from allergic patients for IgE binding validation studies, which typically involve 20 to 50 sera from patients with 0.35 to >100 kU/L IgE antibody to individual allergen molecules.⁶³ This is especially difficult for allergens that are more prevalent in children, such as food allergens. Help is on the way! Smith et al at Vanderbilt University have developed panels of human IgE monoclonal antibodies to dust mite, cat, dog, Aspergillus, and food allergens (peanut, milk, egg, cashew, and walnut). The IgE monoclonal antibodies (mAb) are derived from allergic patients using proprietary technology and, as such, are natural antibodies, of high affinity, that can be produced in mg amounts in cell culture or by recombinant antibody technology.⁶⁹ This remarkable technological breakthrough will, for the first time, enable human IgE mAb to be used for allergen identification, for quantification of IgE antibody responses, for molecular reference standards, for epitope analysis, and in mast cell and basophil

activation assays. In addition to their use in research and allergy diagnostics, human IgE antibodies may have therapeutic applications through defining specific allergenic epitopes that can be modified to produce hypoallergens or to derive specific inhibitors of IgE antibody binding.⁷⁰ Basic research on the IgE repertoire, IgE B-cell transcriptomes, and sequencing of high-affinity allergen specific IgE antibodies will facilitate the development of further functional reagents for potential use in allergy diagnostics.⁷¹⁻⁷⁵

CONCLUSIONS

This review discusses the current state of allergenic extracts in the United States for diagnosis and with licensed products, immunotherapy. The process of standardization is complex, and maintaining high-quality extracts requires extensive work by developers and approval by the FDA. The value and utility of extracts of nonstandardized products depends on the identity of the source material and care in production and storage. Purified or recombinant allergens can be used to provide a more detailed understanding of the individual allergic subject and the proteins that are the dominant allergens for them. As our knowledge builds and with the refinement of methods, we can improve diagnostic accuracy. In the future, we may be able to provide immunotherapy to the specific proteins that are dominant for the individual rather than relying on natural extracts.

REFERENCES

- Hurwitz SH. The development of specialization in allergy. *Calif Med* 1953;78:216-21.
- Brown H, Loveless MH. Allergenic (skin-test) activity of low ragweed pollen after irradiation of extract with ultraviolet light. *Ann Allergy* 1948;6:7-10.
- Johansson SG, Bennich H, Foucard T. Quantitation of IgE antibodies and allergens by the radioallergosorbent test, RAST. *Int Arch Allergy Appl Immunol* 1973;45:55-6.
- Frankland AW. The purity of allergenic extracts. *Dev Biol Stand* 1975;29:101-5.
- Pomés A, Davies JM, Gadermaier G, Hilger C, Holzhauser T, Lidholm J, et al. WHO/IUIS allergen nomenclature: providing a common language. *Mol Immunol* 2018;100:3-13.
- Goodman RE, Ebisawa M, Ferreira F, Sampson HA, van Ree R, Vieths S, et al. AllergenOnline: a peer-reviewed, curated allergen database to assess novel food proteins for potential cross-reactivity. *Mol Nutr Food Res* 2016;60:1183-98.
- Turkeltaub PC, Rastogi SC, Baer H, Anderson MC, Norman PS. A standardized quantitative skin-test assay of allergen potency and stability: studies on the allergen dose-response curve and effect of wheal, erythema, and patient selection on assay results. *J Allergy Clin Immunol* 1982;70:343-52.
- Anderson MC, Baer H. Antigenic and allergenic changes during storage of a pollen extract. *J Allergy Clin Immunol* 1982;69:3-10.
- Miles RM, Parker RL, Jones BS, Dahlberg MJE, Yunginger JW. Studies on *Alternaria* allergens IV. Biologic activity of a purified *Alternaria* fraction (Alt-I). *J Allergy Clin Immunol* 1983;71:36-9.
- Hoffman DR. Immunochemical identification of the allergens in egg white. *J Allergy Clin Immunol* 1983;71:481-6.
- McWilliam V, Peters RL, Allen KJ, Dharmage SC, Ponsonby A-L, Tang MLK, et al. Skin prick test predictive values for the outcome of cashew challenges in children. *J Allergy Clin Immunol Pract* 2020;8:141-8.
- Zhao F, Elkesh A, Durner J, Lindermayr C, Winkler JB, Rueff F, et al. Common ragweed (*Ambrosia artemisiifolia* L.): allergenicity and molecular characterization of pollen after plant exposure to elevated NO₂. *Plant Cell Environ* 2016;39:147-64.
- Zhao F, Durner J, Winkler JB, Traidl-Hoffmann C, Strom T-M, Ernst D, et al. Pollen of common ragweed (*Ambrosia artemisiifolia* L.): illumina-based de novo sequencing and differential transcript expression upon elevated NO₂/O₃. *Environ Pollut* 2017;224:503-14.
- Chen KW, Marusciac L, Tamas PT, Valenta R, Panaitescu C. Ragweed pollen allergy: burden, characteristics and management of an important allergen source in Europe. *Int Arch Allergy Immunol* 2018;176:163-80.
- Visitsunthorn N, Visitsunthorn K, Pacharn P, Jirapongsananuruk O, Bunnag C. Comparison of the efficacy and safety of pollen allergy extracts using skin prick testing and serum specific IgE as references. *Asian Pac J Allergy Immunol* 2017;35:191-5.
- Beneder M, Bublin M, Hoffmann-Sommergruber K, Hawranek T, Lang R. Allergen chip diagnosis for soy-allergic patients: Gly m 4 as a marker for severe food-allergic reactions to soy. *Int Arch Allergy Immunol* 2013;161:229-33.
- Esch RE. Manufacturing and standardizing fungal allergen products. *J Allergy Clin Immunol* 2004;113:210-5.
- Phillips GL. Preparation of allergen extracts for testing and treatment. In: Sheldon JM, Lovell RG, Mathews KP, editors. *A Manual of Clinical Allergy*. Philadelphia: WB Saunders Company; 1953:367-90.
- Giarengo I, Ricciardi T, Alessandri C, Farina L, Crescenzo R, Tuppo L, et al. ENEA, a peach and apricot IgE-binding protein cross-reacting with the latex major allergen Hev b 5. *Mol Immunol* 2019;112:347-57.
- Hauck PR, Williamson S. The manufacture of allergenic extracts in North America. *Clin Rev Allergy Immunol* 2001;21:93-110.
- Bridgewater JL, Slater JE, Rabin RL. Preparation and standardization of allergen extracts. In: Burks AW, Holgate S, O'Hehir R, Bacharier L, Broide D, Hershey GK, et al., editors. *Middleton's Allergy: Principles and Practice*. 9th ed. New York: Elsevier; 2020:467-78.
- Turkeltaub PC. In-vivo standardization. In: Reed CE, Ellis EF, editors. *Allergy, Principles and Practice*. St. Louis: C.V. Mosby Co; 1988:388-401.
- CLSI. Analytical performance characteristics, quality assurance, and clinical utility of immunological assays for human immunoglobulin e antibodies of defined allergen specificities. In: *CLSI Report I/LA20*. 3rd ed. Wayne, PA: Clinical and Laboratory Standards Institute; 2016.
- Hamilton RG, Oppenheimer J. Serological IgE analyses I the diagnostic algorithm for allergic disease. *J Allergy Clin Immunol Pract* 2015;3:833-40.
- Turkeltaub PC. Allergen vaccine unitage based on biological standardization: clinical significance. In: Lockey R, Bukantz SC, editors. *Allergens and Allergen Immunotherapy*. New York: Marcel Dekker, Inc.; 1999:321-40.
- Bonertz A, Roberts G, Slater JE, Bridgewater J, Rabin RL, Hoefnagel M, et al. Allergen manufacturing and quality aspects for allergen immunotherapy in Europe and the United States: an analysis from the EAACI AIT Guidelines Project. *Allergy* 2018;73:816-26.
- Bonertz A, Mahler V, Vieths S. Manufacturing and quality assessment of allergenic extracts for immunotherapy: state of the art. *Curr Opin Allergy Clin Immunol* 2019;6:640-5.
- Slater JE, Menzies SL, Bridgewater J, Mosquera A, Zinderman CD, Ou AC, et al. The US Food and Drug Administration review of the safety and effectiveness of nonstandardized allergen extracts. *J Allergy Clin Immunol* 2012;129:1014-9.
- Bernstein IL, Li JT, Bernstein DI, Hamilton R, Spector SL, Tan R, et al. Allergy diagnostic testing: an updated practice parameter. *Ann Allergy Asthma Immunol* 2008;100(Suppl 3):S1-148.
- Cox L, Nelson H, Lockey R, Calabria C, Chacko T, Finegold I, et al. Allergen immunotherapy: a practice parameter third update. *J Allergy Clin Immunol* 2011;127(Suppl):S1-55.
- U.S. Food and Drug Administration. Prescribing information—GRASSTK. Available from: <https://www.fda.gov/media/88510/download>. Accessed July 19, 2020.
- Matricardi PM, Kleine-Tebbe J, Hoffmann HJ, Valenta R, Hilger C, Hofmaier S, et al. EAACI molecular allergology user's guide. *Ped Allergy Immunol* 2016;(Suppl 23):1-250.
- U.S. Food and Drug Administration. Prescribing information—PALFORZIA. Available from: <https://www.fda.gov/vaccines-blood-biologics/allergenic/palforzia>. Accessed July 19, 2020.
- U.S. Food and Drug Administration. 2011 Meeting Materials, Allergenic Products Advisory Committee. October 25, 2011. Available from: <https://wayback.archive-it.org/7993/20170111224204/http://www.fda.gov/AdvisoryCommittees/CommitteesMeetingMaterials/BloodVaccinesandOtherBiologics/AllergenicProductsAdvisoryCommittee/ucm247212.htm>. Accessed February 10, 2020.
- Ortolani C, Ispano M, Pastorello EA, Ansaloni R, Magri GC. Comparison of results of skin prick tests (with fresh foods and commercial food extracts) and RAST in 100 patients with oral allergy syndrome. *J Allergy Clin Immunol* 1989;83:683-90.
- Nelson HS. Preparing and mixing allergen vaccines for subcutaneous immunotherapy. *Clin Allergy Immunol* 2008;21:303-20.

37. Käck U, Asarnoj A, Grönlund H, Borres MP, van Hage M, Lilja G, et al. Molecular allergy diagnostics refine characterization of children sensitized to dog dander. *J Allergy Clin Immunol* 2018;142:1113-20.
38. Slater JE. Standardized allergen vaccines in the United States. In: Lockett RF, Ledford DK, editors. *Allergens and Allergen Immunotherapy*. 4th ed. Boca Raton, FL: CRC Press, LLC; 2008:273-81.
39. U.S. Food and Drug Administration. Prescribing information—RAGWITEK. Available from: <https://www.fda.gov/vaccines-blood-biologics/allergenics/ragwitek>. Accessed July 19, 2020.
40. U.S. Food and Drug Administration. Prescribing information—ORALAIR. Available from: <https://www.fda.gov/vaccines-blood-biologics/allergenics/oralair>.
41. U.S. Food and Drug Administration. Prescribing information—ODACTRA. Available from: <https://www.fda.gov/vaccines-blood-biologics/allergenics/odactra>. Accessed July 19, 2020.
42. Asarnoj A, Nilsson C, Lidholm J, Glaumann S, Ostholm E, Hedlin G, et al. Peanut component Ara h 8 sensitization and tolerance to peanut. *J Allergy Clin Immunol* 2012;130:468-72.
43. Krause S, Reese G, Randow S, Zennaro D, Quarantino D, Palazzo P, et al. Lipid transfer proteins (Ara h 9) as a new peanut allergen relevant for a Mediterranean allergic population. *J Allergy Clin Immunol* 2009;124:771-9.
44. WHO/IUIS Allergen Nomenclature Sub-Committee. Allergen nomenclature. Available from: <http://www.allergen.org/search.php>. Accessed March 9, 2020.
45. Chapman MD, Pomés A, Breiteneder H, Ferreira F. Nomenclature and structural biology of allergens. *J Allergy Clin Immunol* 2007;119:414-20.
46. Matricardi PM, Dramburg S, Potapova E, Skevaki C, Renz H. Molecular diagnosis for allergen immunotherapy. *J Allergy Clin Immunol* 2019;143: 831-43.
47. Kaul S, May S, Luttkopf D, Vieths S. Regulatory environment for allergen-specific immunotherapy. *Allergy* 2011;66:753-64.
48. Filep S, Tsay A, Vailes LD, Gadermaier G, Ferreira F, Matsui E, et al. Specific allergen concentration of WHO and FDA reference preparations measured using a multiple allergen standard. *J Allergy Clin Immunol* 2012; 129:1408-10.
49. Kaul S, Zimmer J, Dehus O, Costanzo A, Daas A, Buchheit KH, et al. Standardization of allergen products: 3. Validation of candidate European pharmacopoeia standard methods for quantification of major birch allergen Bet v 1. *Allergy* 2016;71:1414-24.
50. Vieths S, Barber D, Chapman M, Costanzo A, Daas A, Fiebig H, et al. Establishment of recombinant major allergens Bet v 1 and Phl p 5a as Ph. Eur. reference standards and validation of ELISA methods for their measurement. Results from feasibility studies. *Pharmazie Bio Sci Notes* 2012;2012:118-34.
51. Pomés A, Chruszcz M, Gustchina A, Minor W, Mueller GA, Pedersen LC, et al. 100 years later: celebrating the contributions of x-ray crystallography to allergy and clinical immunology. *J Allergy Clin Immunol* 2015;136:29-37.
52. Minor W, Cymborowski M, Otwinowski Z, Chruszcz M. HKL-3000: the integration of data reduction and structure solution-from diffraction images to an initial model in minutes. *Acta Crystallogr D Biol Crystallogr* 2006;62(Pt 8):859-66.
53. Chapman MD, Briza P. Molecular approaches to allergen standardization. *Curr Allergy Asthma Rep* 2012;12:478-84.
54. Mindaye ST, Spiric J, David NA, Rabin RL, Slater JE. Accurate quantification of 5 German cockroach (GCr) allergens in complex extracts using multiple reaction monitoring mass spectrometry (MRM MS). *Clin Exp Allergy* 2017;47: 1661-70.
55. Spiric J, Reuter A, Rabin RL. Mass spectrometry to complement standardization of house dust mite and other complex allergenic extracts. *Clin Exp Allergy* 2017;47:604-17.
56. Fenaille F, Nony E, Chabre H, Lautrette A, Couret MN, Batard T, et al. Mass spectrometric investigation of molecular variability of grass pollen group 1 allergens. *J Proteome Res* 2009;8:4014-27.
57. Erler A, Hawranek T, Kruckemeier L, Asam C, Egger M, Ferreira F, et al. Proteomic profiling of birch (*Betula verrucosa*) pollen extracts from different origins. *Proteomics* 2011;11:1486-98.
58. Himly M, Nony E, Chabre H, Van OL, Neubauer A, van Ree R, et al. Standardization of allergen products: 1. Detailed characterization of GMP-produced recombinant Bet v 1.0101 as biological reference preparation. *Allergy* 2009;64: 1038-45.
59. Seppala U, Daulay C, Robinson S, Hornshaw M, Larsen JN, Ipsen H. Absolute quantification of allergens from complex mixtures: a new sensitive tool for standardization of allergen extracts for specific immunotherapy. *J Proteome Res* 2011;10:2113-22.
60. Cromwell O, Hafner D, Nandy A. Recombinant allergens for specific immunotherapy. *J Allergy Clin Immunol* 2011;127:865-72.
61. Valenta R, Karaulov A, Niederberger V, Zhernov Y, Elisyutina O, Campana R, et al. Allergen extracts for *in vivo* diagnosis and treatment of allergy: is there a future? *J Allergy Clin Immunol Pract* 2018;6:1845-55.
62. Posa D, Perna S, Resch Y, Lupinek C, Panetta V, Hofmaier S, et al. Evolution and predictive value of IgE responses toward a comprehensive panel of house dust mite allergens during the first 2 decades of life. *J Allergy Clin Immunol* 2017;139:541-9.
63. Chapman MD, Wuenschmann S, King E, Pomés A. Technological innovations for high-throughput approaches to *in vitro* allergy diagnosis. *Curr Allergy Asthma Rep* 2015;15:36.
64. Chapman MD, Wuenschmann S, King E, Pomés A. Erratum to: technological innovations for high-throughput approaches to *in vitro* allergy diagnosis. *Curr Allergy Asthma Rep* 2015;15:52.
65. Hiller R, Laffer S, Harwanegg C, Huber M, Schmidt WM, Twardosz A, et al. Microarrayed allergen molecules: diagnostic gatekeepers for allergy treatment. *FASEB J* 2002;16:414-6.
66. Lupinek C, Wollmann E, Baar A, Banerjee S, Breiteneder H, Bublin M, et al. Advances in allergen-microarray technology for diagnosis and monitoring of allergy: the MeDALL allergen-chip. *Methods* 2014;66:106-19.
67. Hamilton RG, Hemmer W, Nopp A, Kleine-Tebbe J. Advances in IgE testing for diagnosis of allergic disease. *J Allergy Clin Immunol Pract* 2020;8:2495-504.
68. Gadermaier G, Hauser M, Ferreira F. Allergens of weed pollen: An overview on recombinant and natural molecules. *Methods* 2014;66:55-66.
69. Wurth MA, Hadadianpour A, Horvath DJ, Daniel J, Bogdan O, Goleniewska K, et al. Human IgE mAbs define variability in commercial *Aspergillus* extract allergen composition. *JCI Insight* 2018;3:e12338.
70. Glesner J, Kapingidza AB, Godzwon M, Offermann LR, Mueller GA, DeRose EF, et al. A human IgE antibody binding site on Der p 2 for the design of a recombinant allergen for immunotherapy. *J Immunol* 2019;203:2545-56.
71. Gadermaier E, Levin M, Flicker S, Ohlin M. The human IgE repertoire. *Int Arch Allergy Immunol* 2014;163:77-91.
72. Levin M, Levander F, Palmason R, Greiff L, Ohlin M. Antibody-encoding repertoires of bone marrow and peripheral blood—a focus on IgE. *J Allergy Clin Immunol* 2017;139:1026-30.
73. Looney TJ, Lee JY, Roskin KM, Hoh RA, King J, Glanville J, et al. Human B-cell isotype switching origins of IgE. *J Allergy Clin Immunol* 2016;137:579-86.
74. Croote D, Darmanis S, Nadeau KC, Quake SR. High-affinity allergen-specific human antibodies cloned from single IgE B cell transcriptomes. *Science* 2018; 362:1306-9.
75. Larenas-Linnemann DL, Cox LS. European allergen extract units and potency: review of available information. *Ann Allergy Asthma Immunol* 2008;100:137-45.