

Multiplex for allergy dx: powerful, but it has its place

Amy Carpenter Aquino

December 2018—In allergy testing, microarray technology offers speed and the benefit of smaller sample volumes, but it has a lower sensitivity and is unable to detect IgE antibodies of all specificities in a given extract unless all allergens are on the chip. For routine use, singleplex assays are here to stay.

“Microarray technology will remain a wonderful research tool but will probably not emerge into the clinical world in a serious way for a variety of reasons,” among them a lack of FDA approval, said Robert G. Hamilton, PhD, D.ABMLI, director of the Dermatology, Allergy, and Clinical Immunology Reference Laboratory at Johns Hopkins University School of Medicine.

Dr. Hamilton, who is also a professor of medicine and of pathology at Johns Hopkins, shared a “global perspective of where microarray technology fits into the grand scheme of things,” with its pros and cons, at this year’s American Association for Clinical Chemistry annual meeting.

“The emergence of recombinant components drove us to consider using microarray technology,” he said.

The first allergen chip was developed in 2000 with recombinant allergenic proteins. In 2007, Thermo Fisher Scientific released the primary microarray, the ImmunoCAP immuno solid-phase allergen chip (ISAC), which can test 112 antibody specificities in a single serum analysis. “There have been several publications reporting expanded chip assays with 170 allergenic components and 282 allergenic components and extracts,” he said (Lupinek C, et al. *Methods*. 2014;66[1]:106-119; Heffler E, et al. *World Allergy Organ J*. 2018;11:7).



Dr. Hamilton

Microarray technology is a recent addition to the established “top down” strategy for diagnostic testing, which starts with the patient’s clinical history and examination and is followed by a skin prick test or serological IgE antibody analysis to confirm the specificity of the IgE antibody being present, Dr. Hamilton said. “And as we know, IgE is necessary but not sufficient for eliciting an allergic response and thus generating a definitive diagnosis of allergic disease.”

Allergenic components constitute the second phase in serological testing. “Reflexing to the allergenic components after the specific IgE test with actual extracts is positive is commonplace today,” he said.

While there are pros and cons to the use of allergen extracts, “the bottom-line conclusion is that we will remain using extract-based, solid-phase allergosorbents in IgE antibody serology as a primary tool and expand it using components where it is clinically useful,” Dr. Hamilton said.

Allergen extracts are relatively easy to prepare, and they provide the most comprehensive information for confirmation of sensitization. “They’re physiological extracts and they should contain a comprehensive amount of the allergenic components in that substance specificity.”

“The problem is that they are very heterogeneous and their potency and specificities can sometimes vary between lots and manufacturers,” Dr. Hamilton said. Even global warming is causing changes in some allergen extracts. The level of Amb a 1 in ragweed extracts is increasing, for example, because of rising temperatures and CO2 levels,

which affect the amount of allergenic protein in the pollen that is generated.

Extracts also are highly variable, and there is little quality control. Nineteen of roughly 1,000 extracts used in vivo for diagnosis and immunotherapy are standardized. “Standardized is a soft term when it comes to an extract,” Dr. Hamilton said, “even when the level of an allergenic component is measured in the extract—like Fel d 1 in cat—or a biological allergy unit is assigned based on a biologic response in multiple concentrations of the extract placed on the skin of allergic people.”

Allergenic extracts do not lend themselves to easy differentiation of primary sensitization from a cross-reactivity-driven response because of the complexity of the extract itself, he said. “And you can never predict the relative risk of having a systemic allergic reaction using an extract-based diagnostic test.” Peanut is an example of a case in which reflexing to a group of components (Ara h 1, 2, 3, 6, 8, and 9) makes it possible to form assumptions about the potential risk for a serious reaction in peanut allergic individuals who encounter peanut exposure.

“Diagnostic allergen extracts aren’t perfect but they’re not obsolete,” Dr. Hamilton said. “Running a single allergen extract, let’s say peanut, where in theory it contains most of the allergenic components that are extractable from a peanut, is a good initial test for assessing sensitization in an individual.”

The main strength of the allergen extract is that “it’s a more comprehensive presence of all the allergenic components in that particular specificity versus running a single allergen, where we have to run all the single allergens that are available to cover the one specificity of interest.”

A comparison of allergen extracts and molecular allergens in the areas of purity, potency, availability, quality control, processing artifacts, and comprehensive nature (see table) shows the advantages of molecular allergens. For example, increased regulation of in vivo allergen extracts is limiting their availability while enhanced technology is expanding access to molecular allergens. “Jay Slater’s group at the FDA is identifying a subset of diagnostic and therapeutic allergenic extracts that are being removed because there’s no definitive evidence in the literature that they’re actually efficacious,” Dr. Hamilton said.

One reason for using molecular allergens in IgE antibody diagnostics is concern with the low abundance and/or weak stability of select allergenic molecules. “The illustrative case for me was when we were measuring IgE antibody to hazelnut and all of a sudden it went from very low levels to very high levels with the same specimen,” Dr. Hamilton said.

The manufacturer supplemented the hazelnut extract with Cor a 1, the group 1 allergen in hazelnut that is poorly extracted in a physiological buffer. Recombinant Cor a 1 was added to the hazelnut extract without widely advising customers. “That was the problem,” Dr. Hamilton said. “Our allergists were concerned because all of a sudden they were getting extremely high levels of hazelnut-specific IgE.”

Since Cor a 1 is cross-reactive with Bet v 1, a 17-kilodalton protein from birch pollen, the birch pollen allergic individuals were causing cross-reactive IgE antibody to bind to the hazelnut extract allergosorbent that had been supplemented with Cor a 1. Other examples are adding Gly m 4 in the extract in soy, and omega 5 gliadin in the wheat extract.

“You can take recombinant components and actually supplement the extract and make it a better extract by addressing the concern of the low abundance of certain allergens that are labile,” he said.

Molecular allergens make it possible to better assess the relative risk of selected allergen exposures. “The illustrative case is peanut, where storage proteins Ara h 1, 2, and 3—with Ara h 2 being the best indicator—allow us to make a better assumption that there is a higher risk of having a systemic reaction, versus dealing with sensitization to cross-reactive allergens such as Ara h 8 and 9.”

Cross-reactivity, too, can be assessed. “If we measured Bet v 1 specific IgE in birch pollen allergic individuals, we can expect there to be a broad cross-reactivity with IgE antibody to structurally similar molecules in the PR10

family of proteins,” Dr. Hamilton said.

Finally, molecular allergens are better biomarkers of species-specific sensitization. In hazelnut, for example, Cor a 19 and 14 are much more specific than using hazelnut extract itself on the allergosorbent in terms of assessing the presence of hazelnut sensitivity. Among the primary allergenic families of molecular allergens, Bet v 1 is particularly interesting, he said, citing work by Heimo Breiteneder, PhD, of the Medical University of Vienna. “If you have an IgE antibody to birch or Bet v 1, you can have IgE antibody that binds to structurally similar molecules in a variety of fruit, vegetable, legume, and nut specificities, and it’s tough to distinguish that without actually assessing it at a molecular level.”

Technology for detecting IgE antibody has evolved from isotopic to non-isotopic, from polyclonal to monoclonal anti-IgE, and to the use of heterologous interpolation from a total serum IgE calibration curve. New solid-phase matrix materials have become available with higher binding capacities, so “we’ve moved from the paper disks to the cellulose matrix,” Dr. Hamilton said. Robotics and automation have led to greater assay precision and reproducibility. “We’ve moved from extracts to molecular components on the allergosorbents, and we have seen the emergence of the multiplex technology.”

	Allergen extract	Molecular allergen
Purity	Crude-heterogeneous aqueous extracts	Homogeneous native/recombinant
Lot-potency	Variable	Constant
Availability	In vivo: disappearing in part due to increasing regulations	Expanding availability-enhanced technology
Quality control	Limited	Comprehensive
Processing artifacts	Sensitivity to pH, tissue disruption activates enzymes, solubility issues can lead to missing allergens (lipids)	3D-folding, carbohydrate content, must verify allergenicity immunoreactivity
Comprehensive nature	Strength: comprises most or all allergens from source	Constraint: single allergen specificity

The ImmunoCAP ISAC chip is the predominant IgE antibody multiplex assay. The chip is a glass slide with four sub-squares each containing 112 individual allergenic components, spotted in triplicate, with controls for IgE to ensure the serum is added to the chip.

The assay itself is a standard solid-phase, fluorescent immunoassay where IgE binds with solid-phase allergen. A microarray reader scans the chip and produces an image, which is analyzed and interpolated into a report. Units are measured in semiquantitative ISAC standardized units, or ISUs. “It’s very similar in nature to a singleplex assay, except that you’re simultaneously analyzing IgE antibody to multiple allergens on the actual assay.”

FDA approval is one of the barriers to multiplex analysis, Dr. Hamilton said. “You won’t find chip-based microarrays being run as diagnostic tests in clinical laboratories. They’re great research tools. We use them in a variety of epidemiologic studies, and they are a powerful technology for examining cross-reactivity, but they haven’t arrived yet at federal clearance as a diagnostic test.”

He added: “How would the FDA verify the quality control of 112 individual allergens on a chip? They’re currently exploring ways to validate the performance of even one or two allergens on a chip.”

One of the main strengths of allergen component-based multiplex analysis is its superior ability to identify IgE anti-food/pollen allergen cross-reactivity. It can also detect the presence of relevant IgG- and IgA-blocking antibodies. “Because of the limited amount of allergen on the chip, the IgG antibodies that are present can compete for binding sites with IgE, and this causes an artifactual reduction in the detectable level of IgE antibody,” Dr. Hamilton said. Viennese researchers have suggested the positive aspect of this interference, which is the potential in using the multiplex assay as a tool to examine the effects of immunotherapy where a patient is building up IgG blocking antibody.

The weaknesses of allergen component-based multiplex analysis currently outnumber its strengths: The analytical sensitivity is lower than with singleplex assays, it will not detect IgE antibodies of all specificities in a given allergen extract unless all allergens are on the chip, and IgE binding can be interfered with by antibodies of the same

specificity but different isotypes (“a real negative, in my opinion, in many ways,” he said).

“Most importantly for me is what we preach when we’re teaching the allergists in terms of practice of ordering tests: You order the specificities in which you suspect the individual patient is clinically sensitive, or at least indicates based on a positive history that they are sensitive,” Dr. Hamilton said. “You don’t run 100 allergen specificities as a fixed panel at one time. It’s very inefficient, and you’re producing data you then may have to explain away, because the individual is IgE antibody positive for a particular specificity but has no positive history relevant to that specificity.”

“This panel-testing concept is a concern,” he added. “You can look at it as both a strength and a weakness. It unfortunately encourages abuse of testing.”

The singleplex assay offers performance-related advantages: greater analytical sensitivity, more precise quantification and precision, and established internal and external quality control measures. “It’s much more rigorous versus the multiplex assay’s strengths, which are speed and conservation of the sample, running 30 to 40 microliters to measure 112 individual allergenic specificities.”

The singleplex assay uses a calibration system that is traceable to the third WHO International Standard for serum IgE and has similar units across various assay methods. “Within any given assay, however, it is remarkably reproducible,” he noted. “You can get the same result here in Chicago that you can get in Tokyo because of the reproducibility and stability of the reagents that are used worldwide.”

It also minimizes unneeded testing, is available worldwide, and is more cost efficient in the case of a limited sample number. But the greater need for reagents makes it more costly to run and it requires more technical intervention by staff.

In contrast, the multiplex assay requires fewer reagents and less technician intervention. With its speed and smaller sample volumes, especially beneficial in pediatric testing, “it’s ideal as a point-of-care test.”

A POC assay was on the market briefly. The ImmunoCAP Rapid is a lateral-flow assay that was FDA cleared for 10 aeroallergen specificities. “The assay itself worked well, but the results were being interpreted by the individual patients or the mother and it was a problem,” Dr. Hamilton said. Allergists were concerned, he said, and “laboratories that offered more sophisticated assay testing would miss these additional test requests, but it was a procedure for obtaining a diagnosis of sensitization that was being self-interpreted. Eventually this led to it being removed by the manufacturer from the market.”

Abionic’s IVD Capsule Aeroallergen is a nanotechnology biosensor POC test that mixes serum with fluorescent anti-IgE. The mixture is added to a capsule containing 10 allergens coupled with biosensors on the biosensor surface. Capillary action drives the IgE to immobilize the allergen, and the fluorescent result is measured optically. “Its limitation is the number of specificities, but it’s a novel test.”

Among the proof-of-concept assays is a hydrogel biochip in which 21 allergens, 15 extracts, and six molecules are embedded in gel. The chip has a limited binding capacity, Dr. Hamilton said, and there is limited information about what is binding to the chip.

The multiplex Luminex xMAP-based microarray, designed for indoor aeroallergens, uses an anti-allergen complex on a solid phase. “The assay itself performs well but it’s rather limited,” he said.

Multiplexing systems or reflex singleplex assays using allergenic components offer the greatest potential for natural history and epidemiological studies and for mapping geographically aeroallergen exposure and sensitization patterns. A study published in 2017 examined the geographic distribution of peanut and peanut component specific IgE in a large U.S. population (Valcour A, et al. *Ann Allergy Asthma Immunol.* 119[3]:262-266.e1).

The complex allergen extract has obvious imperfections but “it’s going to be with us for a long time,” Dr. Hamilton said. The challenge is to consider all the patient and environmental factors that drive the IgE antibody response

and look at the four parameters of the immune response that are clinically important: concentration of IgE, affinity, clonality, and specific activity (specific IgE to total IgE ratio). "How many allergists actually measure the total IgE and look at the specific IgE in relation to the total IgE?" he said, noting that this specific activity is a clinically important but overlooked parameter of the immune response.

Whatever the method selected, whether a single-, oligo-, or multiplex assay, "interpretation must be guided by and viewed within the context of the patient's clinical history," Dr. Hamilton emphasizes. And he repeats: "The presence of IgE antibody sensitization is necessary but not sufficient for making the definitive diagnosis of human allergic disease."□

Amy Carpenter Aquino is CAP TODAY senior editor. For additional coverage of the AACC session on allergy testing, see "[Component IgE testing offers food for thought](#)," CAP TODAY, November 2018, www.captodayonline.com.