

Development and Validation of a Third Generation Allergen-Specific IgE Assay on the Continuous Random Access IMMULITE® 2000 Analyzer

Thomas M. Li, Tom Chuang, Susan Tse, Debra Hovanec-Burns, and A. Said El Shami
Diagnostic Products Corporation, Los Angeles, California

Abstract. In vitro determination of allergen-specific IgE (sIgE) represents an important aid in the diagnosis and treatment of allergy. Improvements in laboratory methodology – the development of second, and now third generation assays for sIgE – have brought about major advances in speed, convenience, performance, and in the standards for judging performance. In this study, following the NCCLS I/LA20-A guidelines, we evaluated the analytical performance of a quantitative chemiluminescent enzyme immunoassay for sIgE using the continuous random access IMMULITE® 2000 system. Defining features of this “third generation” sIgE assay include a true zero calibrator with a detection limit and functional sensitivity of 0.1 and 0.2 kU/L, respectively. The use of liquid allergens allows for complete automation, fast binding kinetics between IgE and the natural allergenic protein conformations, and a time-to-first-result of 65 min. Stable reagents and the low nonspecific signal associated with the liquid allergens and centrifugal wash technique permit extension of the measuring range to 0.1–100 kU/L, based on lot-specific, factory-calibrated master curves standardized to the WHO 75/502 reference standard. The assay demonstrated good precision and linearity over its measuring range. Relative to a first generation RIA (mRAST, from Hycor), clinical sensitivity, specificity, and concordance were 88%, 92%, and 90%, respectively ($n = 812$). Quantitative comparisons to a second generation assay yielded a linear regression relationship of $\text{IMMULITE 2000} = 0.99 (\text{Pharmacia FEIA}) + 1.99 \text{ kU/L}$, $r = 0.859$ ($n = 169$). (received 19 October 2003; accepted 27 October 2003)

Keywords: immunoglobulin E, radioallergosorbent test, diagnostic accuracy, NCCLS protocols

Introduction

Allergy is a hypersensitivity reaction initiated by an immunological mechanism [1]. For patients suffering from immunoglobulin E (IgE)-mediated allergy, laboratory-based serological analyses for IgE antibody can be performed, not only as primary confirmation for the diagnosis, but also to identify offending allergens, thus enabling patients to avoid allergens that might trigger allergic reactions – asthma attacks, for example.

Originally described in 1967 by Wide et al [2], the radioallergosorbent test (RAST) was the first assay reported for detection of allergen-specific IgE (sIgE) antibodies. The Phadebas RAST (Pharmacia & Upjohn Diagnostics, Kalamazoo MI) used radioiodinated polyclonal antihuman IgE as signal detection antibody, and the results were reported in arbitrary “Phadebas RAST” units/ml of anti-birch IgE, with a positive/negative cutoff set at 0.35 in these units. In the modified RAST (mRAST) system, which utilizes allergens immobilized on paper discs and two overnight incubations, there is a single calibrator; results are reported in class scores and categorized as positive or negative [3]. The mRAST system yields increased clinical sensitivity, but at the cost of impaired clinical specificity [4,5,6],

Address correspondence to Thomas M Li, Ph.D., Diagnostics Products Corp., 5700 West 96th Street, Los Angeles, CA 90045-5597, USA; tel 310 645 8200, ext 2217; fax 310 645 9999; e-mail tli@dpconline.com. To request a reprint, the catalog number of this article is ZD121.

because only the scoring system is changed, but not the actual detection limit. A quantitative mRAST (Turbo MP, from Hycor) with multiple calibrators was reported by Li et al (American College of Allergy, Asthma and Immunology, 2001 Annual Meeting, Orlando, FL, P-86).

Nonisotopic, second generation RAST-type assays for sIgE [7,8] employ enzyme substrates and photometry or fluorimetry for signal detection. They are quantitative enzyme immunoassays using batch analyzers, with a measuring range of 0.35–100 kU/L, and require approximately 3 hr to complete. They have several advantages over first generation assays, including greater ease of use and a time-to-first-result measured in hr rather than days [9]. Unlike the arbitrary calibration of first generation assays, these assays use calibrators traceable to the WHO IgE reference standard 75/502; nevertheless, they use (nominally) the same cutoff, 0.35 kU/L, for distinguishing positive and negative results. Because the lowest actual calibrator is 0.35 kU/L, which is also true for the quantitative Turbo MP assay, explicit results below this concentration cannot be reported without extrapolation to a “virtual zero calibrator,” that is based on an assumed, rather than measured, dose-response relationship [10].

Responding to perceived clinical need for low assay results reported in the mRAST class system, Diagnostic Products Corporation, Pharmacia, and Hycor have developed the Extended Classification System (ECS), Alternate Scoring Method (ASM), and Turbo Multi-Point System (Turbo MP), respectively, to approximate the mRAST classification. In ASM and Turbo MP, a certain fraction of the signal of the lowest (0.35 kU/L) calibrator serves to classify samples as positive or negative, and to assign mRAST class scores to the positive results. The ASM and Turbo MP both rely on extrapolation below their lowest calibrator [10], in contrast to the ECS, which uses a true zero calibrator.

The aim of the present study was to evaluate the analytical performance of a third generation sIgE assay, using the NCCLS I/LA20-A guidelines [13], and to compare it to representative first and second generation assays. The third generation sIgE assay studied is a chemiluminescent enzyme immunoassay for the quantitation of sIgE in human serum on the

continuous random access IMMULITE® 2000 System [11,12]. This assay has the following characteristics: a substantially broader working range of 0.1 to 100 kU/L with a full calibration curve, including a true zero calibrator; the ability to report results both quantitatively, in kU/L (WHO 75/502), and in standard or extended classes; a functional sensitivity of 0.2 kU/L; the use of liquid allergens, barcoding, and other automation features to reduce labor and human error; and a total assay time of 65 min. The lot-specific, factory-calibrated master curve is highly stable and the manufacturer's recommendations call for 2-point recalibration (“adjustment”) only once every 2 weeks.

Materials and Methods

The IMMULITE 2000 assay (Cat. no. 2KUNG, Diagnostic Products Corp, Los Angeles, CA) utilizes an enzyme-enhanced chemiluminescent enzyme immunoassay for the quantification of sIgE. Streptavidin-coated bead, biotinylated liquid allergen, and patient sample are incubated for 30 min. After a spin wash, an alkaline phosphatase-labeled monoclonal antibody specific for human IgE is added and another 30-min incubation follows. The bead is washed again and the enzyme label is measured with a chemi-luminescent substrate (phosphate ester of adamantyl dioxetane).

In addition to NCCLS guideline I/LA20-A for evaluations of sIgE assays, we followed the NCCLS EP5-A guideline for precision, which describes an experimental design – 20 days, 2 runs/day, 2 replicates/run – yielding estimates of within-run and “total” (interassay) CVs. Precision profiling was applied to the EP5-A CVs to estimate functional sensitivity, defined as the lowest concentration with an interassay CV $\leq 20\%$.

For comparison studies, we used a manual RIA (mRAST, from Hycor Biomedical Inc (Garden Grove, CA) and a fluoroimmunoassay version of the CAP System (Pharmacia & Upjohn Diagnostics, Kalamazoo MI). The former employs a radioisotopic tracer and a single calibrator. The latter has seven classes, with class 0 including all results below 0.35 kU/L and class 6 including all results ≥ 100 kU/L. All assays were performed according to the

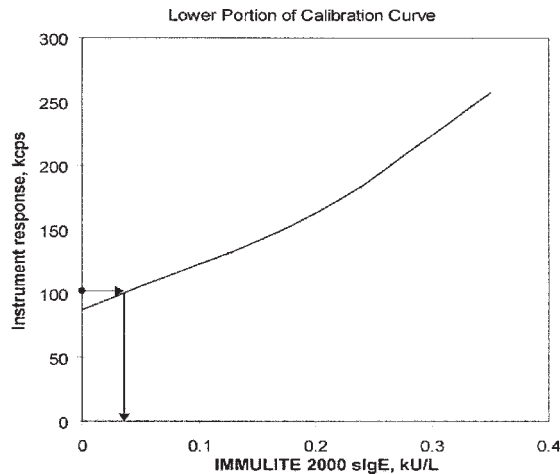


Fig. 1. Low end of a representative calibration ("dose-response") curve for the IMMULITE 2000 sIgE assay, illustrating the results of a detection limit experiment, with the counts (kilo counts/sec, kcps) 2 SD above the average for 20 replicates of the assay's zero calibrator (vertical axis) translated through the calibration curve to the corresponding concentration value (horizontal axis).

manufacturers' instructions in the package inserts (ie, Diagnostic Products Corporation's IMMULITE 2000 package insert, Pharmacia's UniCAP package insert, and Hycor's RIA mRAST package insert).

Results

Detection limit. This was established for the IMMULITE 2000 by assaying replicates of the zero calibrator, using 3 instruments and 20 replicates/instrument. In each of the 3 experiments, the mean and SD were calculated for the counts of the zero calibrator replicates. The counts 2 SD above the mean were translated into concentrations via the calibration curve, yielding an estimate of the assay's detection limit in kU/L, in accord with the classic NCCLS-recommended experimental design for this performance measure [13].

The process is illustrated in Fig. 1. All 3 estimates were <0.1 kU/L, the IMMULITE 2000 assay's conservatively stated detection limit. Note, for contrast, that a second generation assay for sIgE, because it lacks a zero calibrator, must treat the concentration of its lowest calibrator, ie, 0.35 kU/

Table 1. Within-run precision for IMMULITE 2000 sIgE assay.

Number of samples	Concentration ranges (kU/L)	Median % CV
8	Very low (0.2 - 0.875)	10.7
8	Low (0.875 - 1.75)	5.8
28	Medium (1.75 to 7)	4.9
15	High (>7)	4.6

L, as the assay's limit of detection or quantitation [14]. This important parameter cannot be determined experimentally for such an assay.

Precision and functional sensitivity. Precision characteristics of the IMMULITE 2000 sIgE assay were determined according to the NCCLS EP5-A guideline in 2 studies, performed 12 mo apart. The EP5 design yields estimates of within-run CVs and "total" CVs for each sample. The latter correspond more closely to the interassay CVs relevant in actual practice: the CVs that are routinely monitored by clinical laboratories via internal QC procedures.

Table 1 summarizes the within-run CVs observed for the IMMULITE 2000. These meet the NCCLS I/LA20-A performance targets for within-run imprecision, namely < 10% at "low," "medium," and "high" concentrations. Also tabulated are CVs at "very low" concentrations – down to 0.2 kU/L, which is below the range where second generation assays can yield quantitative results without extrapolation. Fig. 2 shows "total" (interassay) CVs generated in the 2 EP5 experiments. These meet NCCLS I/LA20 A performance targets for "total" imprecision, <15% at "medium" or "high" concentrations and <20% at "low" concentrations, ie, close to 0.35 kU/L.

Also shown in Fig. 2 is a curve fitted to the EP5 "total" CVs. This "precision profile" – which represents the interassay CVs for the IMMULITE 2000 sIgE assay as a function of concentration across its working range – indicates a "functional" sensitivity of 0.2 kU/L for the assay. Although originally developed for TSH [15], the concept of functional sensitivity and its operational definition in terms of a 20% interassay CV have been widely adopted for other analytes, as a means of indexing the concentration range over which an assay can yield

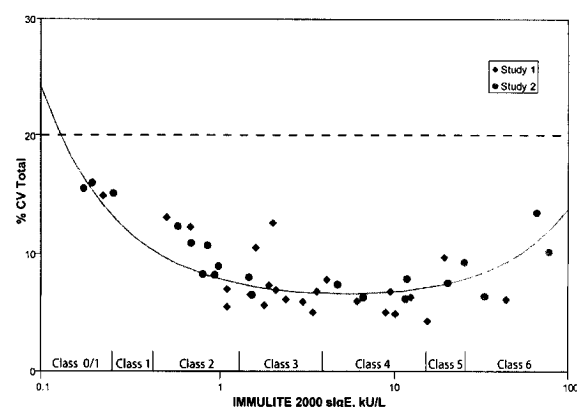


Fig. 2. Total ("interassay") CVs from two separate NCCLS EP5 precision studies of the IMMULITE 2000 sIgE assay, fitted with a precision profile. The allergens included D1, D2, E1, E5, F13, F14, G6, H2, K82, M2, M6, and T3, as well as an IgE control. The relationship between extended classes (ECS) and sIgE concentration (in kU/L) is indicated along the horizontal axis. A horizontal dotted line indicates the 20% interassay CV level used to define "functional sensitivity."

clinically useful results. Accordingly, the functional sensitivity of an assay is defined as the lowest concentration at which it yields an interassay CV of $\leq 20\%$. This concept has gained acceptance as an objective indication of the practical lower limit of an assay's usefulness; it is more relevant than indices based on within-run CVs or detection limits.

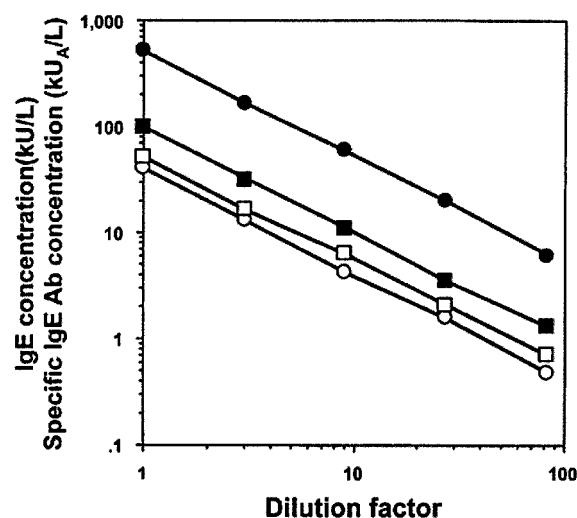
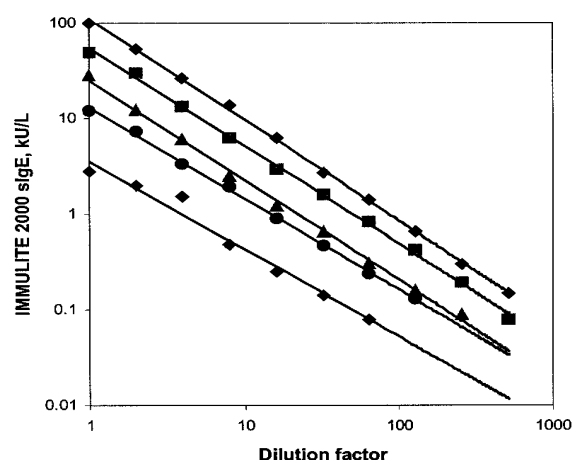
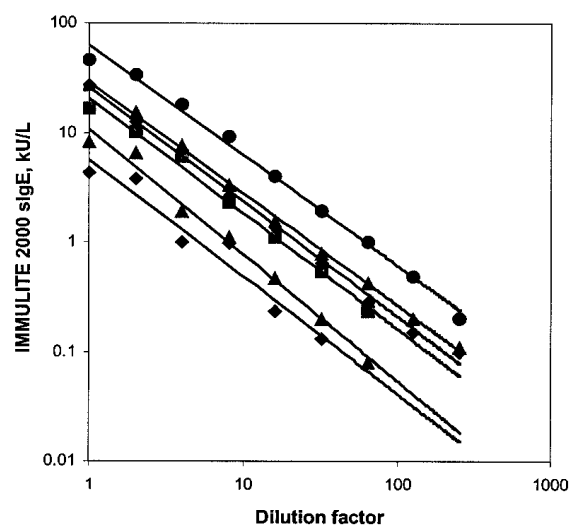


Fig. 3. Dilutional linearity for a representative second generation sIgE assay, using both samples and calibrators. From Yunginger et al [17]. Reproduced with permission of the publisher (Elsevier).



Figs. 4 and 5. Dilutional linearity for the IMMULITE 2000, a third generation sIgE assay. From top to bottom: F8, M2, D3, I1, W1, K82 (Fig. 4) and Calibrator, G2, E1, T6, F33 (Fig. 5).

Dilutional linearity. Fig 3 shows the linearity-under-dilution performance of a representative second generation sIgE assay on samples and calibrator [16]. The dilution factors used were between 1 and 100, extending down only to 0.5 kU/L. Figs. 4 and 5 show results for the IMMULITE 2000 sIgE assay, illustrating the broader range of dilutional linearity that can be achieved by a third generation assay. The dilution factors were between 1 and 1000, extending

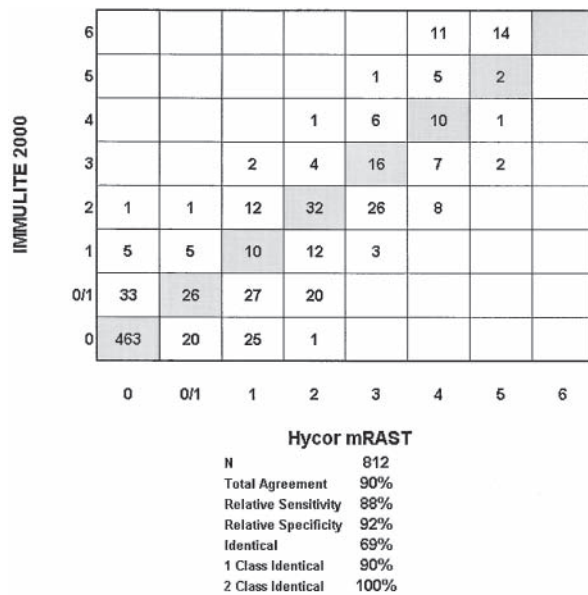


Fig. 6. Comparison of the IMMULITE 2000 to the manual Hycor mRAST, a first generation sIgE assay, in terms of extended class scores.

down to 0.1 kU/L, the assay's conservatively stated detection limit (which is well below the standard 0.35 kU/L cutoff).

Interference. Serum samples ($N = 7$) containing varying amounts of sIgE (1.4 to 64 kU/L) for E1, E5, T3, G6, G2, M6 and D1 were spiked with increasing amounts of bilirubin, hemoglobin, or triglycerides. Spiked and unspiked samples were then assayed by the IMMULITE 2000. Bilirubin (up to 20 mg/dl), hemoglobin (up to 500 mg/dl), and triglycerides (up to 3000 mg/dl) did not have any significant effect on the sIgE results.

Crossreactivity. Subclasses of naturally occurring immunoglobulins (IgA, IgD, IgG, and IgM) were spiked into the IMMULITE 2000 sIgE assay's zero calibrator. (All dilutions were at least 1 in 20.) With % crossreactivity defined as 100 times the apparent concentration, divided by the amount added, crossreactivity with IgA, IgD, IgG, and IgM proved $<0.001\%$ in each case, as recommended in the NCCLS I/LA20 A guidelines.

Nonspecific binding. Three samples containing high total IgE levels (greater than 1000 kU/L) were tested with 22 different allergens, including 2 dusts, 1 animal, 5 foods, 5 molds, 3 trees and 6 weeds. The IMMULITE 2000 sIgE results were all below 0.1 kU/L, the assay's detection limit.

Method comparisons. The IMMULITE 2000 sIgE assay yields quantitative results in kU/L, but these can be translated into either standard or extended class scores. Fig. 6 shows a comparison of the IMMULITE 2000 sIgE assay and the manual Hycor mRAST in terms of extended class scores. Forty-eight serum samples from atopic individuals containing different levels of antibodies to a variety of allergens were obtained from a serum bank. There were a total of 812 paired results, representing 66 allergens, including 5 animals, 1 dust, 10 foods, 7 grasses, 4 insects, 2 mites, 8 molds, 15 trees, and 14 weeds. Relative to the mRAST, clinical sensitivity, specificity, and concordance were 88%, 92%, and 90%, respectively. All of the paired results agreed within ± 2 classes, most (90%) agreed within ± 1 class, and over two-thirds (69%) of the samples yielded identical class scores. The comparison demonstrates good agreement between the automated IMMULITE 2000 sIgE and the manual Hycor mRAST in terms of class scores, despite the methodological differences between the two assay systems.

In another study, the IMMULITE 2000 sIgE assay was compared – in quantitative terms, rather than classes – to the Pharmacia FEIA, a second generation assay. Forty-nine serum samples from atopic donors in another serum bank were tested for 17 different allergens – including D1, D2, E1, E2, E5, F1, F3, F4, F14, F76, F77, F78, G6, K82, M6, T3, and W6 – for a total of 169 paired results between 0.35 and 100 kU/L by both methods. Linear regression analysis yielded the following relationship: IMMULITE 2000 = 0.995 (Pharmacia FEIA) + 1.986 kU/L, $r = 0.859$, $n = 169$. Fig 7 shows that the paired results are fairly evenly distributed above and below the "line of identity." The comparison demonstrates good agreement between the automated IMMULITE 2000 sIgE and the Pharmacia FEIA in terms of concentration (kU/L), despite methodological differences.

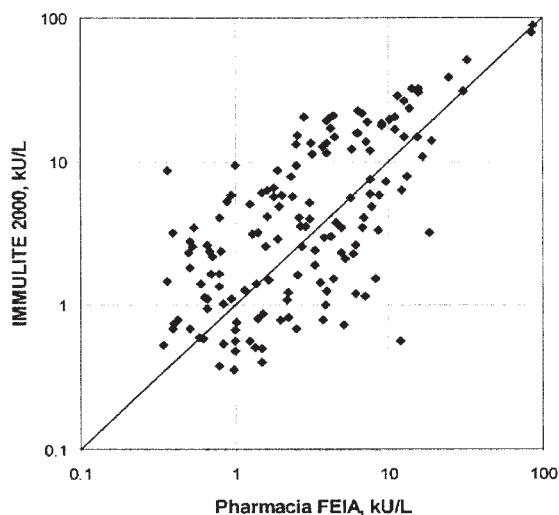


Fig. 7. Comparison of the IMMULITE 2000 to the Pharmacia FEIA, a second generation sIgE assay, in terms of kU/L. The diagonal line represents the "line of identity."

Discussion

At present, 3 manufacturers offer sIgE assays with claims that the tests are quantitative (Diagnostic Products Corporation's IMMULITE 2000 package insert, Pharmacia's UniCAP package insert, and Hycor's Turbo mRAST package insert). All use calibration traceable to the WHO IgE reference standard 75/502.

The first generation RAST assay requires 2 overnight incubations, and the second generation FEIA provides results in 3 hr, whereas the third generation IMMULITE 2000 sIgE assay has a time-to-first-result of about 65 min.

Second generation assays lack a true zero calibrator: the lowest calibrator has a concentration of 0.35 kU/L. Although the Turbo MP does permit the reporting of explicit results below 0.35 kU/L, these are obtained by extrapolation, from percentage-of-signal at 0.35 kU/L, down to a concentration range not supported by the calibration curve (Fig. 8). If a second generation assay simply reports such results as <0.35 kU/L, then the assay reverts, in effect, from quantitative to ASM class-based reporting of results obtained by extrapolation,

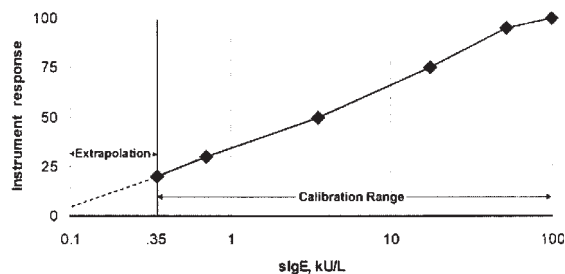


Fig. 8. Illustration of extrapolation and assumed (virtual) zero calibrator and cutoff response in second generation sIgE assays (such as Turbo MP and ASM). Note that the IMMULITE 2000 sIgE assay uses two calibrators below 0.35 kU/L, including a true zero calibrator.

below the concentration of its lowest calibrator. With the recent emphasis on early detection of antibodies against allergens [17], explicit, calibration curve-based information on sIgE concentrations less than 0.35 kU/L may be of clinical interest because allergen avoidance can significantly reduce both the degree of sensitization and the development of allergic symptoms [18].

The third generation sIgE assay studied in this paper overcomes the limitations exhibited by first and second generation sIgE assays, including the lack of a true zero calibrator, experimentally undefined assay detection limit, and poor precision and accuracy [19]. The third generation IMMULITE 2000 sIgE assay has immunoassay design features such as liquid phase allergens, monoclonal detection antibody, enzyme-enhanced chemiluminescent signal detection; use of an actual (rather than a virtual) zero calibrator, allowing explicit results as low as 0.1 kU/L to be reported by interpolation (rather than extrapolation) from the calibration curve; a detection limit of 0.1 kU/L; a functional sensitivity of 0.2 kU/L; dilutional linearity down to 0.1 kU/L; and "user convenience" and efficiency features such as continuous random access automation and a time-to-first-result of approximately one hr. Many advantages of this assay are derived from its detection system based on enzyme-enhanced chemiluminescence, which is orders of magnitude more sensitive than other conventional methods of signal detection [20].

Table 2. Characteristics of the three generations of specific IgE assays.

Features	First generation	Second generation	Third generation
Allergens	Solid phase, on paper disc	Solid phase, on cellulose sponge	Liquid phase allergens
Detection antibody	Polyclonal	Monoclonal	Monoclonal
Signal detection	Isotopic	Fluorescent or spectrophotometric	Enzyme-enhanced chemiluminescent
Results reported in	"Arbitrary" units, and classes	WHO-standardized kU/L, and classes	WHO-standardized kU/L, and classes
Lowest calibrator	25 kU/L	0.35 kU/L	0.0 kU/L
Zero calibrator	No	No	Yes
Detection limit	N/A	0.35 kU/L by default	0.1 kU/L by experiment
Functional sensitivity	Not determined	Not determined	0.2 kU/L
Dilutional linearity	N/A	Down to 0.35 kU/L	Down to 0.1 kU/L (below the standard 0.35 kU/L cutoff)
Automation level	Manual	Batch	Continuous random access
Time-to-first-result	2 overnights	3 hr	65 min

Table 2 lists the characteristics of the 3 generations of sIgE assays. Benefits of the fully automated, third generation IMMULITE 2000 sIgE assay include an extended working range; a lower, well-defined detection limit; improved precision in the neighborhood of the standard 0.35 kU/L cutoff; and reductions in allergen and sample setup time with a reduced time-to-first-result of 65 min, instead of multiple hr or days.

Although the NCCLS I/LA20-A guidelines [13] were issued in 1997, the current study represents, to our knowledge, the first published report in which these guidelines have been used for complete validation of the analytical performance of an sIgE assay. Evaluation parameters detailed in NCCLS I/LA20-A include linear range, within-run (intra-assay) imprecision, total (interassay) imprecision, detection limit, dilutional linearity, nonspecific binding, correlation to other methodologies, calibration curve stability, interferences, and crossreactivity. The IMMULITE 2000 assay meets all the performance targets set for the NCCLS I/LA20-A evaluation criteria. The clinical performance of this sIgE assay in comparison with skin testing will be reported elsewhere (Ollert M et al, in prepar-

ation), as will interlaboratory and intermethod comparisons (Fu PC, Zic V, in preparation), and a multicenter precision profile study (Oosterom R, in preparation).

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