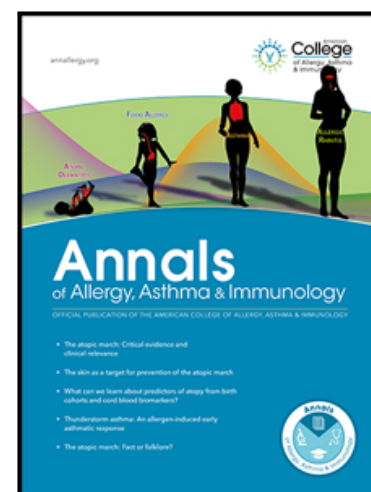


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Validation of the ALEX microarray platform for specific IgE detection of respiratory and plant-food allergens

Short title: Validation of the ALEX microarray platform

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Conflict of Interest:

The authors have no conflicts of interest to disclose.

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Keywords: Component-resolved diagnosis. Microarray. Specific IgE. Respiratory allergens. Food allergens. Reproducibility. Linearity.

Contributors:

PQ, CMDA and MJG equally contributed to study design. PQ, CMDA, MP, BEG, GG, NBL, MIA, FJF, CM, JB, MF and MJG contributed to participant recruitment for clinical performance assessment, and PQ, MSB, MP and MJG performed the technical analysis. PQ, MSB, CMDA and MJG organized data collection, analysis, and figure preparation. PQ, MSB, CMDA, MP, BEG, GG, NBL, MIA, FJF, CM, JB, MF and MJG contributed to the literature search and writing of the original draft of the manuscript and its critical revision.

Abbreviations:

ALEX	Allergy Explorer, by Macroarray Diagnostics
CCDs	Cross-reactive carbohydrate determinants
CRD	Component-resolved diagnosis
CV	Coefficient of variation
FEIA	Fluorescence enzyme immunoassay
ICC	Intraclass coefficient correlation
IgE	Immunoglobulin E
IQR	Interquartile range
ISAC	Immuno Solid-phase Allergen Chip
ISU	ISAC Standard Units
LTP	Lipid transfer protein
RUO	Research Use Only
Se	Sensitivity
Sp	Specificity

Background: As the use of multiplex specific IgE (sIgE) detection methods becomes increasingly widespread, proper comparative validation assessments of emerging new platforms are vital.

Objective: To assess the clinical and technical performance of a newly introduced microarray platform, ALEX (MacroArray Diagnostics), in the diagnosis of pollen (cypress, grass, olive), dust mite *Dermatophagoides pteronyssinus*, *Alternaria alternata*, fruit (apple, peach) and nut (walnut, hazelnut and peanut) allergy, and to compare it to that of the ImmunoCAP ISAC 112 microarray and the ImmunoCAP singleplex method (ThermoFisher Scientific).

Methods: We enrolled 153 allergic patients and 16 non-atopic controls. sIgE assays were conducted using ISAC112, ALEX version 2 (ALEX2), and ImmunoCAP for whole extracts and major components. Technical validation of ALEX2 was performed by measuring repeatability and inter-assay, inter-batch and inter-lab reproducibility.

Results: When measured globally (detection by one or more allergen components), ALEX2 showed adequate sensitivity and specificity for most of the allergens studied, comparable in general to that of ISAC112 (except for olive pollen and walnut) and similar to that of ImmunoCAP whole extract measurements. Component-by-component analysis showed comparable results for all techniques, except for Ole e 1 and Jug r 3 in both ISAC112 and ImmunoCAP comparisons, and Alt a 1, when compared with ISAC112. Continuous sIgE levels correlate with sIgE by ImmunoCAP. Good reproducibility and repeatability were observed for ALEX2.

Conclusion: ALEX2 shows sound technical performance, and adequate diagnostic capacity, comparable in general to that of ISAC112 and ImmunoCAP.

Introduction

The use of multiplex platforms in component-resolved diagnosis (CRD) has aided allergists in obtaining detailed information on a patients' specific allergenic profile by means of a single-measurement method. However, some limitations have been uncovered for the first commercially available allergen platforms.^{1, 2} Furthermore, adequate panel selection^{3, 4} and the inclusion of whole extract detection as a complement to component analysis, have shown to be essential for an adequate and cost-effective diagnosis.^{5, 6} A new microarrayed-allergen platform, named Allergy Explorer (ALEX, MacroArray Diagnostics), has recently been introduced. By means of nanoparticle-based technology, it performs a quantitative analysis of specific IgE (sIgE) to a combination of natural and recombinant allergen components, as well as whole extracts. Its diagnostic capacity seems to correlate well with that of two previously established techniques, the ImmunoCAP ISAC⁷⁻⁹ and ImmunoCAP singleplex method.⁹⁻¹¹

This study aims to assess the clinical performance of ALEX version 2 (ALEX2) in the diagnosis of pollen (cypress, grass, olive), dust mite (*Dermatophagoides pteronyssinus*), mold (*Alternaria alternata*), fruit (apple, peach) and nut (walnut, hazelnut and peanut) allergy. We also sought to compare its diagnostic capacity with the ImmunoCAP ISAC 112 microarray (ISAC112) and ImmunoCAP. Finally, we aimed to perform a technical validation studying ALEX2's repeatability, reproducibility and quantification capacity by employing linearity assessment.

Methods

Patients

We included 169 subjects: 153 allergic patients to cypress, grass or olive pollen, *D. pteronyssinus*, *A. alternata* and/or allergic to apple, peach, walnut, hazelnut and/or peanut, and 16 non-atopic controls. Most patients (77%) were selected from a previous multicentric study (Grant: PI 11/01634) which evaluated the diagnostic performance of the ISAC 112 (ThermoFisher Scientific) microarray in a sample from 14 hospitals from different areas in Spain.^{1, 3, 12} New eligible patients were recruited at Clínica Universidad de Navarra in Pamplona, Navarra (Spain) for dust mite or nut allergy, and at Hospital Clinic in Barcelona (Spain) for *Alternaria* allergy. Inclusion/exclusion criteria are detailed in eMethods. The study protocol was approved by the Ethics Committee of the coordinating center (045/2011), and all participants provided written informed consent.

Multiplex Specific IgE Assay

All sera were tested using ALEX2 (44 allergens) and ISAC112 (33 allergens) for the 10 sources evaluated (eTable 1). Total and sIgE against the described allergens were evaluated using the ALEX2 platform according to manufacturer's instructions (detailed in eMethods). The Raptor software (MacroArray Diagnostics, Vienna, Austria) calculates the levels of IgE based on the intensity of precipitated color in the membrane according to a calibration curve measured in kU/L for values between 0.30 and 50 kU_A/L. Total IgE is also measured for values between 20 and 2500 kU/L. Values of sIgE equal to or greater than 0.30 kU_A/L were considered positive, following manufacturers' instructions. For clinical validation, ALEX2 was performed using the automatic robot Madmax (MacroArray Diagnostics).

Specific IgE against the described allergens (eTable 1) were measured in all patients using ISAC112 (ThermoFisher, Uppsala, Sweden), according to manufacturer's instructions (detailed in eMethods). Specific IgE values are expressed semiquantitatively as ISAC Standard Units (ISU).

Results equal to or greater than 0.30 ISU were considered positive, as per manufacturer's instructions.

Singleplex Specific IgE Assay

For each evaluated biological source and for both cases and controls, sIgE levels to the whole extract and to the major allergen components in our area (eTable 1) were determined by fluorescence enzyme immunoassay (FEIA) (ImmunoCAP, ThermoFisher). Cup a 1 was detected to be the major allergen of *Cupressus arizonica*, as were Phl p 1, Ole e 1, Der p 1 and Der p 2, Alt a 1, Mal d 3, Pru p 3, Jug r 3, Cor a 8 and Ara h 9 as major allergens of *Phleum pratense*, *Olea europea*, *D. pteronyssinus*, *A. alternata*, apple, peach, walnut, hazelnut and peanut respectively (based on previous studies).^{1, 3} In addition, Der p 23 was measured for *D. pteronyssinus* cases and controls. Specific IgE values equal to or higher than 0.35 kU_A/L by FEIA were considered positive.

Technical validation

Repeatability was assessed using intra-assay analysis, and reproducibility was assessed using inter-assay, inter-batch and inter-laboratory analysis for the 44 evaluated allergens (eTable 1). ALEX2 repeatability was tested by analyzing a pool of sera in 10 measurements performed in the same assay, with the same ALEX2 kit (same batch), in one laboratory. Reproducibility of the platform was assessed by analyzing the inter-assay sIgE variability from 10 samples (9 individual sera and the pool), and by repeating the ALEX2 technique in 5 different days, using chips from the same production batch, in the same laboratory. Inter-batch reproducibility was also evaluated by analyzing sIgE levels from the 10 samples using two different chips and reagents from different production batches. Finally, inter-laboratory reproducibility was studied by analyzing the levels of sIgE obtained from the 10 samples using chips from the same batch (reagents from different batches) analyzed in two different laboratories: Clínica Universidad de Navarra (Pamplona, Spain)

and Hospital Clínic (Barcelona, Spain). The reading of the chips was performed in each laboratory according to manufacturer's instructions.

To explore ALEX2's quantitative sIgE determination capacity, serial dilutions of several sera were analyzed. ALEX2 test was performed on the same day, using the same batch in the same laboratory, using the pool of sera and 3 sera as described: concentrated, 1:2, 1:5 and 1:10, diluted in water. ALEX2 technique was performed manually for technical validation and blocking cross-reactive carbohydrate determinants (CCDs) interference according to manufacturer's instructions.

Statistical Analysis

Variables were evaluated for normal distribution (Shapiro-Wilk test). Non-normal quantitative values were described as medians and interquartile ranges (25-75 percentile). Qualitative values were described as frequencies (percentages). Proportions were compared using the Chi² test or Fisher exact test. Ordinal and quantitative variables were analyzed using the U Mann Whitney test. Qualitative results comparing performance of two techniques for the same sample were analyzed by McNemar's test. Concordance between variables was analyzed using the Kappa index (κ) and was interpreted to be poor (<0.2), weak (0.21-0.4), moderate (0.41-0.6), good (0.61-0.8), or very good (0.8-1) according to the Altman model.¹³ The Wilcoxon signed rank test was used for quantitative comparison in paired samples. The correlation between the quantitative variables was evaluated using the Spearman correlation coefficient. Clinical statistical analysis was performed using Stata/IC 12.0. Differences of $P < 0.05$ were statistically significant.

We also analyzed the strength of agreement of the results obtained for all 44 allergens. Repeatability of these results under the same conditions, was assessed by calculating the global intraclass coefficient correlation (ICC), and reproducibility of the results obtained in the different assays (inter-assay, inter-batch and inter-lab) under different conditions, was evaluated by calculating the ICC for each allergen. The level of agreement using the ICC was expressed using

the Fleiss classification:¹⁴ very good (ICC>0.90), good (0.71–0.90), moderate (0.51–0.70), mediocre (0.31–0.50) or poor (ICC<0.30). For the pooled sera, sIgEs to the studied allergens were also analyzed individually by calculating the coefficient of variation (CV) for positive results (allergens showing positive results in at least 50% of the 10 repeated measurements) in the intra-assay analysis and in the inter-assay analysis (for allergens showing positive results in at least 50% of the 5 repeated measurements). Also, we analyzed the reliability of the technique comparing the number of allergens that modified the positivity/negativity of the IgE according to the range established by the manufacturer (0.3 kUA/L), in the intra-assay, inter-assay, inter-batch and inter-lab measurements for each serum. The ICC for each allergen were performed using the statistical software package SPSS (Statistical Package for social science) for Windows, version 20. The CV was calculated using Excel 2016, Microsoft Office. Linearity was assessed by analyzing slope and R^2 with simple linear regression and comparing serial diluted sIgE data with expected values for all allergens showing basal results equal to or above 3 kUA/L. Linearity analysis and graphs were performed in GraphPad Prism 9.0.

Results

Patient characteristics

From 153 allergic patients and 16 nonatopic controls, cases and controls were selected for each allergen employing the specified criteria. Thus, for the aeroallergen diagnostic performance analysis of the microarray, we included 28 cases and 26 controls (50% non-atopic) for cypress pollen, 29 cases and 27 controls (48% non-atopic) for grass pollen, 27 cases and 31 controls (52% non-atopic) for olive pollen, 20 cases and 22 controls (59% non-atopic) for *D. pteronyssinus*, and 18 cases and 22 controls (45% non-atopic) for *A. alternata*. For food allergens, apple was represented by 15 cases, peach by 34, walnut by 28, hazelnut by 20 and peanut by 19 cases, together with 23 controls (69.6% non-atopic). eTable 2 sets out the clinical and demographic data of the individuals included in the study, grouped into cases and controls for allergy diagnosis to respiratory (eTable 2A) and food allergens (eTable 2B).

Global diagnostic performance of the ALEX2 microarray is adequate for the majority of the studied allergens

When considering positivity of one or all components representing the evaluated biological source as diagnostic of allergy, ALEX2 yielded good performance for cypress pollen, grass, *D. pteronyssinus*, *A. alternata*, apple, peach, hazelnut and peanut. Improvable sensitivity (Se) was observed for olive pollen and walnut allergy (Table 1). Olive pollen's Se was increased when Fra e 1, the major allergen of the Oleaceae ash pollen, was considered in the calculations (Se: 77.8%, specificity (Sp): 93.5%). In fact, higher levels of sIgE to Fra e 1 [3.07 (0.48-19.23) kU_A/L] than for Ole e 1 [0.76 (0-5.47) kU_A/L] were observed in olive pollen cases (Wilcoxon test $P < 0.001$) by ALEX2, with 81.5% of olive allergic patients being from areas without ash pollen relevant levels.

Diagnostic performance of the ALEX2 microarray is quite similar to ISAC microarray for the evaluated allergens

Component-based sensitization profiles in cases and controls were also assessed using the ISAC112 multiplex platform for the selected allergens (Table 2). ALEX2's global diagnostic capacity was compared to that observed for ISAC112, considering the components present there for the same evaluated biological sources. Sensitivity and specificity for both platforms is presented in Table 1. ISAC112 showed comparable performance to ALEX2 for cypress pollen, grass pollen, *D. pteronyssinus*, peach, hazelnut and peanut allergy detection. ISAC112 Se showed to be higher for the detection of olive pollen and walnut allergies, while it was significantly lower for the detection of *Alternaria* and apple allergies.

When analyzing data on a component-by-component basis for those elements common to both the ALEX2 and ISAC112 platforms, ALEX2 showed a similar diagnostic performance compared to ISAC112 in all of them except for Ole e 1, Alt a 1 and the under-research allergen Ole e 7; Jug r 3 tended to show a worse performance by ALEX2 than by ISAC112 but differences did not reach statistical significance. Comparative data are shown in Table 2.

Diagnostic performance of ALEX2 microarray is quite similar to ImmunoCAP for whole extract/components of the evaluated allergens

sIgE to whole extracts and to major allergens of the evaluated allergenic sources was also measured using the singleplex ImmunoCAP. ALEX2's overall diagnostic capacity was compared to that shown for the selected sources whole extracts by ImmunoCAP (Table 2). Similar diagnostic performance was observed for most of the evaluated allergens (cypress pollen, grass pollen, *D. pteronyssinus*, *A. alternata*, apple, peach, hazelnut and peanut). Se for ImmunoCAP whole extract was significantly superior than for ALEX2 in the diagnosis of olive pollen and walnut allergy. Se for sIgE to *A. alternata* by ImmunoCAP was not compared with ALEX2, since our inclusion

criteria included positivity of the *A. alternata* whole extract allergen when considering patients as “*Alternaria* allergic”.

Moreover, when analyzing data on a component by-component basis for selected allergens (major allergens in our sample) by ALEX2 and ImmunoCAP, ALEX2 showed similar diagnostic performance for all the allergens tested; Jug r 3 tends to show worse performance by ALEX2 than by ImmunoCAP but these differences do not reach statistical significance (Table 2). In addition, in the knowledge that the ALEX2 platform has been created as a quantitative method, correlation analysis was performed using sIgE against selected major components between ImmunoCAP and ALEX2, showing high correlation coefficients, all of them over 0.750. Given that ImmunoCAP and ALEX2 results are both measured in kU_A/L, quantitative comparisons were performed for sIgE to these major components resulting in higher sIgE levels by ALEX2 than by ImmunoCAP for 7 of the 12 allergens in cases and higher sIgE by ImmunoCAP than ALEX2 in 7 of the 12 allergens in controls (Table 3).

Total IgE

Taking into consideration that total IgE is measured semiquantitatively by ALEX2, a quantitative correlation analysis was performed for total IgE measured by ALEX2 and ImmunoCAP. Total IgE values obtained in ALEX2 below 20 and above 2500 kU/L were not included, since the platform cannot detect them precisely, and neither were total IgE values by ImmunoCAP below 2 and above 5000 kU/L, according to manufacturer’s specifications. Thus, 163 patients (147 cases and 16 controls) were finally used for the analysis. A good correlation was observed between both techniques (Spearman Rho: 0.8114; $P < 0.001$) (eFigure 1).

Technical validation

The global repeatability of the ALEX2 array was found to be excellent, 0.9979 (alpha Crombach). Variability of the repeated measurements for the studied allergens (intra-assay analysis) was also analyzed individually by calculating the coefficient of variation (CV) for positive results (allergens with a median value ≥ 0.3 kU_A/L: 32 out of 44 allergens) for the pooled sera. CV data were grouped in ranges based on sIgE levels of each allergen and median intra-assay CV were calculated for each range (Table 4). All allergens with values over 1 kU_A/L showed CVs under 15%, while higher CVs were observed for values between 0.3 and 1. As the group size for this range was very small, we also analyzed its CV using all allergens in the array which showed positive sIgE between 0.3 and 1 kU_A/L (N=50). When doing so, the actual median CV was 20.8% (13.7-27.9% IQR). Finally, when considering all 44 allergens, repetitions were consistent for all of them in terms of sensitization results (positive/negative) except for Alt a 6, for which half of the results were positive and half were negative, showing a high variability (CV: 107%, median, range: 0.29, 0.10-1.66 kU_A/L) and Der p 21, which showed only one result above 0.30 kU_A/L (CV: 100%, median, range: 0.09, 0.00-0.31 kU_A/L). Two other allergens which showed positive and negative results in the repeated measurements, presented moderate variability and data scattered around the positivity cut-off value: Phl p 5.0101 (CV: 29%, median, range: 0.37, 0.21-0.55 kU_A/L), and Phl p 6 (CV: 38%, median, range: 0.41, 0.23-0.85 kU_A/L).

Reproducibility was assessed individually for the studied allergens using two approaches: the CV calculated for positive results in the inter-assay analysis (allergens with a median value ≥ 0.3 kU_A/L: 33 out of 44) for the pooled sera and ICC for 10 different samples (pooled sera and 9 samples). CV were grouped based on sIgE ranges of sIgE levels and median inter-assay CV were calculated for each range (Table 4). As expected, slightly higher variability was observed for pooled sera measurements conducted in different days, compared with repetitions in the same day, but median CV for allergens with values over 1 kU_A/L was also under 15% (Table 5). Once more, when

evaluating the CV in the low range (0.3-1 kU_A/L), we had a small group size and thus, we also analyzed it with all eligible allergens in the array (N=48) to obtain more robust data. In this case, the actual median CV for the low range was 22.47% (14.9-31% IQR). Again, most of the allergens depicted consistent results in terms of sensitization outcome (positive/negative results), except for Alt a 6 (CV: 108%, median, range: 0.32, 0.10-1.89 kU_A/L), Ara h 15 (CV: 183%, median, range: 0, 0-0.71 kU_A/L) and Der p 5 (CV: 93%, median, range: 0.39, 0-0.55 kU_A/L). Two other allergens showing positive and negative results in different measurements presented moderate variability and data close to the cut off value: Phl p 5.0101 (CV: 36%, median, range: 0.27, 0.26-0.53 kU_A/L) and Phl p 6 (CV: 39%, median, range: 0.36, 0.28-0.69 kU_A/L). Considering the consistency of data between different samples, in general, high ICC were observed for inter-assay determinations (Table 5).

Inter-batch and inter-lab variability were calculated by analyzing two measurements under different conditions: different batch and different laboratory, respectively. ICC was calculated for inter-batch and inter-laboratory analysis (Table 5), including the pool, along with 9 samples. Regarding consistency of data, from 440 determinations evaluated in inter-batch analysis (44 allergens in 10 sera), only 17 (3.9%) depicted differences that led to a different sensitization diagnostic (positive/negative sIgE). Moreover, from 440 determinations evaluated in the inter-laboratory analysis, only 11 (2.5%) depicted differences that led to a different sensitization diagnosis.

Linearity analysis was performed for those allergens showing measurements equal or over 3 kU_A/L, to ensure dilutions fell within the positive value range of the technique. Using this criterion, we included 59 measurements in this analysis, covering 28 of our 44 studied allergens. Linearity of sIgE by ALEX2 platform has been reported,¹⁵ with an upper limit of detection described at 50 kU_A/L. We observed that, for allergens with high sIgE concentrations (>30 kU_A/L), there was a decrease of the slope and the regression coefficient (R^2) values (Figure 1), suggesting poor linearity in the upper range. For concentrations lower than 30 kU_A/L and higher than 0.3 kU_A/L, most of the

measurements depicted good linearity. Within this concentration range, only 6 linear regressions out of 48 showed very poor metrics (Figure 1), including Alt a 6 (analyzed in one sample), Ara h 3 (in two of two analyzed samples), Cup a 1 (in one sample of four analyzed samples), Mal d 1 (in one sample of two analyzed samples) and Pru p 3 (in one sample of four analyzed samples). To note, Alt a 6 showed the worst linearity data, consistently depicting a deficient performance in our technical analysis.

Discussion

Allergy diagnosis has largely benefited from multiplex allergen platforms based on molecular components. Ever since the Immuno-Solid Phase Allergen Chip (ISAC) was introduced twenty years ago,¹⁶ other techniques have been developed, each one with its own set of allergens and technical features.^{17,18} Our study sought to assess the diagnostic performance of one of the newest platforms, ALEX version 2 (ALEX2), comparing it to the gold standard in sIgE quantification, ImmunoCAP, and the most widely used multiplex array, ImmunoCAP ISAC 112, in a properly characterized sample.

In general, the ALEX2 platform shows sound capacity for the detection of patients allergic to some aeroallergens, certain fruits, and nuts, proving to be a reliable tool for molecular diagnosis. ALEX2 diagnostic performance is quite similar to that of the previous existing platforms, ISAC112 and ImmunoCAP, for most of the studied allergens. However, exceptions to the above statement are evident in the capacity for detection of olive pollen-allergic patients, and walnut-allergic individuals based on Jug r 3 performance. When interpreting data pertaining to olive pollen component detection, a low sensitivity was found in general. Sensitivity is much improved when adding to the analysis Fra e 1, a molecular component of the ash tree pollen (an Oleacea tree) that shows high identity with Ole e 1.¹⁹ Fra e 1 seems to expose relevant olive pollen epitopes better than the Ole e 1 molecule in the ALEX2 chip. Until the Ole e 1 molecule is improved, it may be wise to consider Fra e 1 in olive pollen diagnosis when interpreting ALEX2 results in cases showing a clinical

profile compatible with olive pollen allergy, it may be of value to consider. Moreover, although ALEX2 has extended the spectrum of walnut allergens represented in the chip, and Jug r 1 and Jug r 2 seem to diagnose similarly to those in ISAC112, detection of allergic patients is strengthened via Jug r 3, since our is predominantly sensitized to the walnut lipid transfer protein (LTP).

ALEX2 showed improved diagnostic capacity for *Alternaria* and apple allergies compared with ISAC112, due to the better performance of Alt a 1 and the inclusion of Mal d 3, respectively. It is important to consider that in terms of CRD, extensively wide panels of allergens allow the inclusion of clinically relevant allergens such as Der p 23,^{9, 20} absent in ISAC112. In fact, 15% of *D. pteronyssinus* s allergic patients showed sensitization to this component without sensitization to the classic major allergens, being misdiagnosed by ISAC112 (analyzing Der p 1, Derp 2, Derp 10). The addition of whole extracts to the sample component panels in these new platforms, also reported in other platforms,⁵ seems to increase sensitivity for certain allergenic sources as well. Even though ALEX2 includes whole extract detection for some allergens, these extracts belong to biological sources other than those considered in our analysis.

ALEX2's diagnostic capacity seems to be quite similar to that of ISAC112 in terms of qualitative data. Furthermore, ALEX2 is a quantitative technique showing good correlation with sIgE by ImmunoCAP. In terms of equivalence of sIgE, different levels of sIgE have been detected using ALEX2 and ImmunoCAP. Since ImmunoCAP has shown a good agreement between the use of ng/mL and kU_A/L units as a measurement of sIgE by external laboratories as well,²¹ we believe a similar external validation should be performed for ALEX2 and for different allergens and concentrations. We noted that the quantification in the upper scale of the dynamic range (over 30 kU_A/L) depicted poor linearity, suggesting that the actual dynamic range is lower than 50 kU_A/L. This has been recently reported by some authors, who suggest that the dynamic range is between 0.30 and 30-40 kU_A/L, in line with our findings.^{8, 11} Nevertheless, we consider that low linearity in

these value ranges is not of significant relevance, as values this high are not frequent, and linear quantification at this point is of low clinical consequence.^{22, 23}

Total IgE detection is comparable between ALEX2 and ImmunoCAP. Given the importance of considering total IgE when interpreting sIgE results, this may represent a cost-effective improvement over ISAC112, which requires a separate ImmunoCAP singleplex assay in order to measure total IgE.²⁴

When conducting our technical analysis, we observed that the technique exhibits good reproducibility and repeatability performance in our hands. As shown with intra- and inter-assay variability data, the technique generally provides robust sIgE values. Higher variability was observed in lower range values, since CV is highly influenced by mean value, something that has been also noted for ISAC 112.²⁵ Nevertheless, CV was quite high for some allergens such as Alt a 6. The technique also proved to be robust in the inter-batch and inter-laboratory assessment, where most of the allergens depicted excellent results and with a very low percentage of sensitization data disagreements. As a limitation, we acknowledge that our technical analysis is not based on all the allergens available in the array, focusing only on a set of allergens of interest. Thus, overall results could slightly vary.

As previously commented by other authors,⁷ the ALEX2 microarray contains a broader panel of allergens to evaluate sensitizations using a top-down approach, offering a global test at a lower cost per patient than the ISAC 112. In addition, ALEX2 can be automatized, thus reducing the effect and need of human-handling and shortening the time taken to run each test when compared with ISAC 112. Therefore, ALEX2 emerges as a promising tool, especially in polysensitized patients. After validation of all the allergens present in ALEX2, cost-effectiveness analysis studies comparing different available techniques should be performed.

In conclusion, according to our study results, ALEX2 is a microarray showing adequate diagnostic capacity, comparable to that of the ISAC112 multiplex and ImmunoCAP singleplex, showing sIgE

ImmunoCAP-correlated quantitative results up to 30 kAU/L. Furthermore, repeatability and reproducibility of the ALEX2 platform has been demonstrated. The spectrum of application of our results is limited to patients with Mediterranean sensitization profiles, so further evaluations are recommended in other regions for patients with varying allergenic exposures.

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Tables and Figures:

Figure 1. Graphical presentation of the linearity analysis in 4 samples, covering 28 of 44 studied allergens (59 determinations). The graph plots R^2 values (from linear regression analysis with the serial dilutions) (X-axis) and allergen initial sIgE concentrations (Y-axis). Allergens with an initial concentration <30 kU_A/L and a $R^2 < 0.9$ are identified.

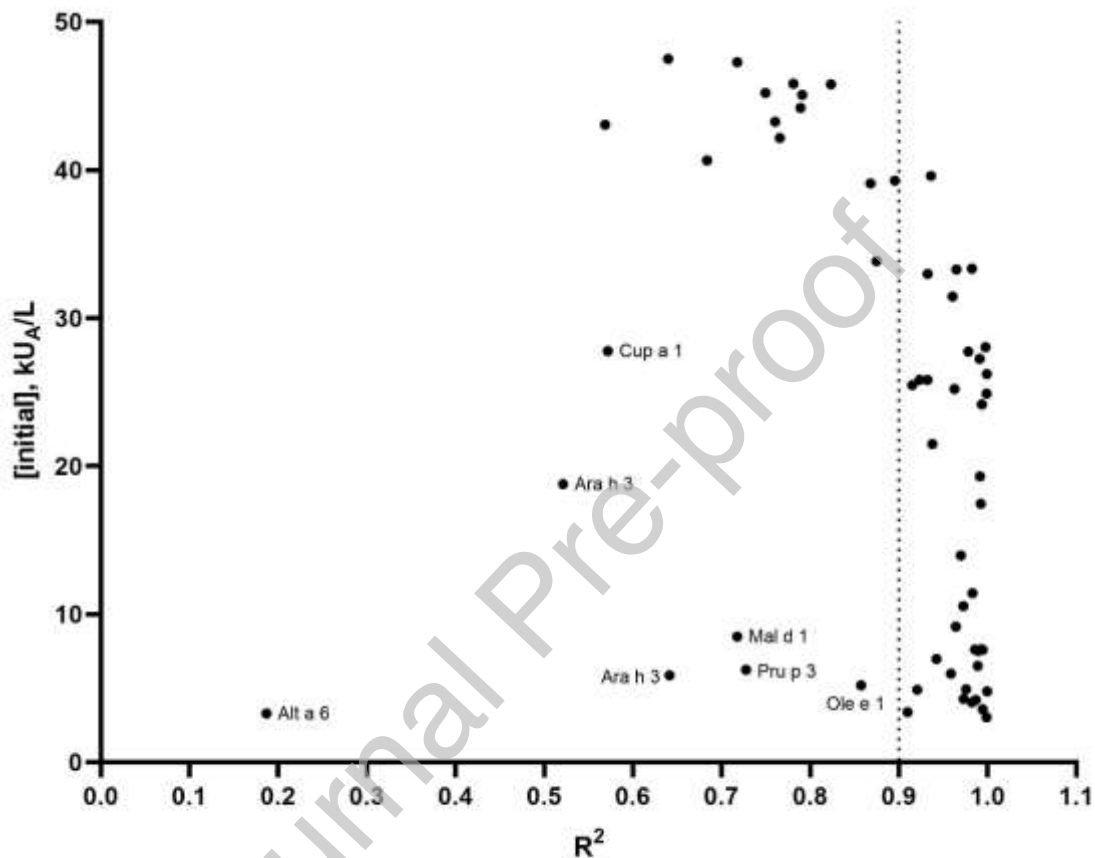


Table 1. Sensitivity (Se) and specificity (Sp) of sIgE in the diagnosis of allergy using ALEX2, ISAC112 and ImmunoCAP. Positivity to all or one of the components representing the biological source was regarded as diagnostic for ALEX2 and ISAC112, while positivity to whole extract was considered for ImmunoCAP. *P-values*<0.05 marked in bold.

Biological source		ALEX2			ISAC112		ALEX2 vs ISAC112 McNemar test p		Whole extract ImmunoCAP		ALEX2 vs ImmunoCAP McNemar test p
		Se	Sp		Se	Sp			Se	Sp	
Cypress pollen	Cup a 1	85.7%	96.2%	Cup a 1	85.7%	92%	1.000	Cup a	82.1%	96.2%	1.000
Grass pollen	Phl p 1, 2, 5.0101, 6, 7, 12	100%	88.9%	Phl p 1, 2, 4, 5, 6, 7, 11, 12	96.5%	88.9%	1.000	Phl p	96.4%	92.3%	0.500
Olive pollen	Ole e 1, 7, 9	55.5%	96.8%	Ole e 1, 7, 9	85.2%	90.3%	0.0020	Ole e	92.6%	87.1%	0.0002
Dust mite	Der p 1, 2, 5, 7, 10, 11, 20, 21, 23	95%	100%	Der p 1, 2, 10	80%	100%	0.250	Der p	85%	100%	0.625
Alternaria	Alt a 1, 6	88.9%	100%	Alt a 1, 6	55.6%	100%	0.0313	Alt a	†	100%	0.500
Apple	Mal d 1, 2, 3	73.3%	100%	Mal d 1	6.7%	100%	0.0020	Mal d	100%	100%	0.125
Peach	Pru p 3, 7	94.1%	100%	Pru p 1, 3, 4	97.1%	100%	1.000	Pru p	100%	100%	0.500
Walnut	Jug r 1, 2, 3, 4, 6	60.7%	100%	Jug r 1, 2, 3	85.7%	95.7%	0.0215	Jug r	92.9%	100%	0.0117
Hazelnut	Cor a 1.0401, 8, 9, 11, 12, 14	100%	91.3%	Cor a 1.0104, 8, 9	90%	100%	0.125	Cor a	100%	100%	0.500
Peanut	Ara h 1, 2, 3, 6, 8, 9, 15	94.7%	95.7%	Ara h 1, 2, 3, 6, 8, 9	84.2%	100%	0.250	Ara h	100%	100%	1.000

† Positive sIgE to *A. alternata* by ImmunoCAP was an inclusion criterion for Alternaria cases, and thus Se has not been calculated.

Table 2. Frequency of sensitization to the components from the studied sources present in ALEX2, ISAC112 and ImmunoCAP, for cases and controls. Qualitative comparisons are represented in terms of positive/negative results between ALEX2 sIgE and ImmunoCAP/ISAC112 sIgE to different components. Negative results by all techniques were not analyzed. *P-values*<0.05 marked in bold.

% Sensitization		ALEX2		ImmunoCAP		ALEX2 vs. Immuno CAP	ISAC112		ALEX2 vs. ISAC112
Biological source and components		Cases	Controls	Cases	Controls	κ (<i>P</i> Mc Nemmar)	Cases	Controls	κ (<i>P</i> Mc Nemmar)
Cypress pollen	Cup a 1	85.7%	3.8%	82.1%	3.8%	0.963 (1)	85.7%	8%	0.962 (1)
Grass pollen	Phl p 1	96.6%	11.1%	96.4%	7.1%	0.893 (0.25)	93.1%	3.7%	0.928 (0.5)
	Phl p 2	48.3%	3.7%				37.9%	3.7%	0.854 (0.25)
		Phl p 5.0101					Phl p 5		
	Phl p 5	37.9%	3.7%				34.5%	3.7%	0.945 (1)
	Phl p 6	34.5%	3.7%				24.1%	3.7%	0.811 (0.25)
	Phl p 7	6.9%	3.7%				3.4%	3.7%	0.811 (1)
	Phl p 12	17.2%	0%				13.8%	3.7%	0.780 (1)
Olive pollen	Ole e 1	55.6%	3.2%	66.7%	6.5%	0.839 (0.125)	74.1%	9.7%	0.734 (0.015)
	Ole e 7	0%	0%				22.2%	0%	0.000 (<0.001)
	Ole e 9	3.7%	0%				0%	0%	0.000 (1)
<i>D. pteronyssinus</i>	Der p 1	60%	0%	70%	0%	0.889 (0.5)	60%	0%	1 (1)
	Der p 2	70%	0%	70%	0%	1 (1)	65%	0%	0.946 (1)
	Der p 5	40%	0%						
	Der p 7	55%	0%						
	Der p 10	0%	0%				10%	0%	0.000 (0.5)

	Der p 11	0%	0%						
	Der p 20	0%	0%						
	Der p 21	35%	0%						
	Der p 23	85%	0%	70%	4.7%	0.842 (1)			
Alternaria	Alt a 1	88.9%	0%	100%	0%	1 (1)	55.6%	0%	0.6667 (0.031)
	Alt a 6	0%	0%				11.1%	0%	0.000 (0.5)
Apple	Mal d 1	6.7%	0%				6.7%	0%	0.493 (1)
	Mal d 2	0%	0%						
	Mal d 3	66.7%	0%	86.7%	0%	0.691 (0.375)			
Peach	Pru p 1						0%	0%	
	Pru p 3	94.1%	0%	96.9%	4.3%	0.927 (0.5)	97.1%	0%	0.893 (1)
	Pru p 7	0%	0%						
Walnut	Jug r 1	14.3%	0%				14.3%	0%	1 (1)
	Jug r 2	17.9%	0%				17.9%	4.4%	0.694 (1)
	Jug r 3	46.4%	0%	64.3%	0%	0.771 (0.063)	67.9%	0%	0.642 (0.07)
	Jug r 4	10.7%	0%						
	Jug r 6	14.3%	0%						
Hazelnut	Cor a 1.0401	5%	4.3%				5%	0%	0.656 (1)
	Cor a 8	90%	0%	90%	0%	1 (1)	80%	0%	0.903 (0.5)
	Cor a 9	5%	0%				5%	0%	1 (1)
	Cor a 11	5%	4.3%						
	Cor a 12	0%	0%						
	Cor a 14	10%	0%						
Peanut	Ara h 1	15.8%	0%				15.8%	0%	1 (1)

	Ara h 2	15.8%	0%				15.8%	0%	1 (1)
	Ara h 3	10.5%	0%				5.3%	0%	0.656 (1)
	Ara h 6	15.8%	0%				15.8%	0%	0.641 (1)
	Ara h 8	0%	0%				0%	0%	NA
	Ara h 9	84.2%	0%	84.2%	0%	1 (1)	73.7%	0%	0.897 (0.5)
	Ara h 15	0%	4.3%						

NA: Not analyzed

Table 3. Specific IgE levels (kU_A/L) against the major allergens measured by ALEX2 and by ImmunoCAP, for cases and controls. Median and interquartile range (IQR) are presented. Spearman correlation between the two techniques was calculated combining cases and controls together. Spearman correlation Rho coefficients and *p*-values are presented.

		ALEX2 [kU _A /L, median (IQR)]		ImmunoCAP [kU _A /L, median (IQR)]		ALEX2 vs ImmunoCAP Spearman correlation	
Biological source		Cases	Controls	Cases	Controls	<i>Rho</i>	<i>p</i>
Cypress pollen	Cup a 1	7.45 (1.39-17.33)	0.05 (0-0.12)	3.84* (0.82-9.88)	0* (0-0.02)	0.890	<0.001
Grass pollen	Phl p 1	6.72 (4.35-23.2)	0 (0-0.06)	4.01* (2.03-9.55)	0 (0-0)	0.915	<0.001
Olive pollen	Ole e 1	0.76 (0-5.47)	0 (0-0)	0.67** (0.08- 7.74)	0** (0-0.03)	0.895	<0.001
<i>D. pteronysinus</i>	Der p 1	3.3 (0-9.45)	0 (0-0)	2.33 (0.02-10.65)	0 (0-0)	0.856	<0.001
	Der p 2	21.25 (0.15-33.43)	0.03 (0-0.08)	7.19* (0.04-18.5)	0* (0-0)	0.853	<0.001
	Der p 23	5.39 (1.13-10.4)	0 (0-0)	1.47* (0.17-4.97)	0** (0-0.02)	0.810	<0.001
<i>A. alternata</i>	Alt a 1	28.05 (8.86-36.03)	0 (0-0)	8.59* (2.59-12.5)	0 (0-0)	0.944	<0.001
Apple	Mal d 3	1.79 (0.09-7.95)	0 (0-0)	2.73 (1.2-3.95)	0** (0-0)	0.834	<0.001
Peach	Pru p 3	5.39 (2.55-15.64)	0 (0-0)	6.17 (2.35-12.2)	0** (0-0)	0.928	<0.001
Walnut	Jug r 3	0.17 (0.01-2.16)	0 (0-0.01)	1.03 (0.1-2.38)	0.01** (0- 0.02)	0.799	<0.001
Hazelnut	Cor a 8	2.09 (1.41-9.09)	0.06 (0.03-0.14)	1.19* (0.66-2.7)	0* (0-0)	0.824	<0.001
Peanut	Ara h 9	3.93 (2.28-10.99)	0.1 (0.02-0.73)	2.29* (1.23-6.23)	0* (0-0.01)	0.780	<0.001

* Wilcoxon signed rank test $P < 0.05$ between sIgE by ALEX2 and ImmunoCAP showing higher sIgE by

ALEX2 than by ImmunoCAP for cases and controls.

*** Wilcoxon signed rank test $P < 0.05$ between sIgE by ALEX2 and ImmunoCAP showing higher sIgE by ImmunoCAP than by ALEX2 for cases and controls.*

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Table 4. Intra-assay and inter-assay coefficient of variability (CV) obtained with the pool of sera. Allergens were classified in ranges according to their median sIgE. Median CV and interquartile ranges (IQR) were calculated for each group (number of allergens used reported). Only allergens with a median value ≥ 0.3 kU_A/L were considered.

Median sIgE range (kU _A /L)		Median intra-assay CV, % (IQR)		Median inter-assay CV, % (IQR)
<0.30	(N=12)		(N=11)	
≥ 0.30 - <1	(N=4)	27.6 (23.7-30.9)	(N=5)	39.1 (14.4-93.4)
≥ 1 - <10	(N=16)	6.5 (5.1-8.2)	(N=15)	11 (8.5-14.9)
≥ 10	(N=12)	4.5 (3.1-7.1)	(N=13)	8.1 (7.2-14.4)

Table 5. Inter-assay (N=5 determinations), inter-batch (N=2 determinations) and inter-lab (N=2 determinations) intraclass coefficient of variability obtained for the 44 allergens evaluated. ICCs below 0.7 for allergens showing positive mean sIgE are marked in bold.

Allergen		Inter-assay ICC	Inter-batch ICC	Inter-lab ICC
Cypress pollen	Cup a 1	0.996	0.998	1
Grass pollen (<i>Phleum pratense</i>)	Phl p 1	0.995	0.965	0.99
	Phl p 2	0.993	0.996	0.999
	Phl p 5.0101	0.973	0.977	0.992
	Phl p 6	0.972	0.983	0.983
	Phl p 7	0.998	0.878	0.999
	Phl p 12	0.986	0.763	0.995
Olive pollen	Ole e 1	0.996	0.998	0.981
	Ole e 7 RUO	0.773†	-†	-0.179†
	Ole e 9	0.988	0.963	0.998
<i>A. alternata</i>	Alt a 1	0.998	1	0.99
	Alt a 6	0.915	0.684	0.841
<i>D. pteronyssinus</i>	Der p 1	0.998	0.997	1
	Der p 2	0.999	0.999	1
	Der p 5	0.999	0.998	1
	Der p 7	0.997	0.680	1
	Der p 10	0.6	-0.385†	-0.274†
	Der p 11	0.678	-0.372†	-0.78†
	Der p 20	0.995	0.987	0.998
	Der p 21	0.998	1	1
	Der p 23	0.999	0.998	1
Apple	Mal d 1	0.998	0.987	0.979
	Mal d 2	0.884	-0.88	-0.399†
	Mal d 3	0.989	0.948	0.991
Peach	Pru p 3	0.972	-0.390	0.998

	Pru p 7 RUO	0.233†	0.737†	0†
Hazelnut	Cor a 1.0401	0.981	0.966	0.969
	Cor a 8	0.993	0.996	0.998
	Cor a 9	0.989	0.958	0.994
	Cor a 11	0.989	0.999	0.999
	Cor a 12 RUO	0.047†	-0.175†	0†
	Cor a 14	0.999	0.965	0.997
Peanut	Ara h 1	0.999	0.975	0.99
	Ara h 2	0.999	0.989	0.99
	Ara h 3	0.989	0.981	0.808
	Ara h 6	0.998	0.988	0.999
	Ara h 8	0.97	0.932	0.861
	Ara h 9	0.989	0.754	0.996
	Ara h 15	0.848	0.446	0.61†
Walnut	Jug r 1	0.999	0.996	0.999
	Jug r 2	0.999	0.995	0.969
	Jug r 3	0.989	0.976	0.987
	Jug r 4	0.993	0.998	0.998
	Jug r 6	0.998	0.990	0.995

ICC: intraclass coefficient correlation, RUO: research use only

† Allergen depicted negative mean sIgE values in all the samples of the analysis (<0.30 kU_A/L)