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Performance of a polymer coated silicon microarray for simultaneous detection of food allergen specific IgE and IgG₄

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Abstract

Background: Microarray-based component-resolved diagnostics (CRD) has become an accepted tool to detect allergen specific IgE-sensitization towards hundreds of allergens in parallel from one drop of serum. Nevertheless specificity and sensitivity as well as a simultaneous detection of allergen specific IgG₄, as a potential parameter for tolerance development, remain to be optimized.

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Objective: We applied the recently introduced silicon chip coated with a functional polymer named copoly(DMA-NAS-MAPS) to the simultaneous detection of food allergen specific IgE and IgG₄, and compared it with ImmunoCAP and ImmunoCAP ISAC. Inter- and intra- slide variation, linearity of signal and working range, sensitivity and application of internal calibrations for IgE and IgG₄ were assessed.

Methods: Native and recombinant allergenic proteins from hen's egg and cow's milk were spotted on silicon chips coated with copoly(DMA-NAS-MAPS) along with known concentrations for human IgE and IgG₄. A serum pool and 105 patient samples were assessed quantitatively and semi-quantitatively with the ImmunoCAP and ImmunoCAP ISAC and correlated with IgE- and IgG₄-specific fluorescence on silicon microarrays.

Results: Allergen specific IgE and IgG₄ were detected in parallel using two fluorescent dyes with no crosstalk. Results from the ImmunoCAP correlated better with microarray fluorescence than with ImmunoCAP ISAC except for the allergen ovomucoid. The working range of the silicon microarray for total hen's egg specific IgE was comparable to the range of 0.1 to >100 kU_A/l of the ImmunoCAP system, whereas for total cow's milk the silicon microarray was less sensitive. Detectable allergen specific IgG₄ could be determined only for low concentrations, but still correlated positively with ImmunoCAP results.

Conclusions: We confirmed the ability of the polymer coated silicon microarray to be comparably sensitive to the ImmunoCAP ISAC for various food allergens. This suggests that the copoly(DMA-NAS-MAPS) microarray is a low cost, self-producible alternative to the commercial ImmunoCAP ISAC in allergy research.

Introduction

Protein microarrays have become a well-acknowledged tool in bio-analytical and clinical applications since their introduction (1, 2). Up to thousands of peptides and proteins, immobilized onto a small area, can be analyzed with only a small volume containing the ligands of interest to be captured. Beside application in cancer and drug research, microarrays have proven to be of great value in allergic diseases (3-7). Assumed to be even more sensitive than macroscopic bioassays (8), this technique is of special interest in component resolved diagnostics (CRD) of allergic diseases where small concentrations of IgE (given in kU/l; 1 kU/l corresponding to 2.42 µg/l) against individual allergenic molecules

of food and aeroallergens must be detected in small volumes of patient samples. One of the most common platforms in routine allergy diagnostics is the ImmunoCAP (Thermo Fisher), a single-plex system with a working range from 0.1 to 100 kU_A/l. Allergen-specific IgE is considered the most important clinical parameter to diagnose allergy, although the relation between clinical reactivity and level of IgE is still not fully understood (9).

Despite intense research on surface chemistry and detection techniques (10-14), accurate quantification of captured IgE in kU_A/l (A for allergen-specific) on microarrayed platforms for the diagnosis of food allergy is still not optimal. Reliability, sensitivity and correlation with established routine platforms (15, 16) need to be improved. Since protein microarrays have to struggle with maintenance of protein structure on their surface, a lot of effort has been invested in the development of a surface that has a higher binding capacity and increased stability of fragile proteins (17, 18). Some findings have suggested high sensitivity and precise quantification of IgE, but the works were mostly focused on inhalant allergens or used small numbers of sera (19-21). The application of microarrays in high throughput clinical allergy diagnostics is still limited by calibration issues (22), costs and lack of automation.

The most common microarray detection platform in research for component-resolved diagnostics (CRD), the ImmunoCap ISAC (Thermo Fisher), is based on 112 allergens from 51 sources spotted in triplets on standard glass slides. Captured IgE antibodies are detected by fluorescent-labelled secondary antibodies and their concentration is expressed in semi-quantitative Internal Standardized Units (ISU) according to an external calibration serum. Either IgE-or IgG₄-specific signals can be detected in a 30 µl sample volume (serum or plasma), diluted 1:2 or 1:50, respectively. ISAC has been used as a reference in several studies to evaluate novel platforms (23, 24).

With the introduction of a silicon/silicon oxide (Si/SiO₂) substrate (25), coated with the functional polymer copoly(DMA-NAS-MAPS) (26), we have provided a highly sensitive microarray platform with better reproducibility compared to glass slides whose performance was demonstrated in the analysis of sera of 30 adults with sensitization to inhalant allergens (27). The improved performance of Si/SiO₂ slides with a controlled oxide layer of 100 nm is the result of enhanced fluorescence intensity detected on the surface thanks to constructive optical interference (28).

In the present study, we assessed the performance of copoly(DMA-NAS-MAPS)-coated silicon slides using well characterized sera of children with sensitization to hen's egg and cow's milk and correlated those to results found using ISAC and ImmunoCAP.

Material and Methods

Patient sera

Several sets of sera were used as listed in **Table 1**. Set A was comprised of 91 well-characterized sera from a previous study (29). These sera were used for comparison of quantitative (ImmunoCAP) and semi-quantitative (ImmunoCAP ISAC) results with fluorescence from the silicon microarray for extracts and components. From these 91 sera, 15 samples were selected with sIgE to CM covering ImmunoCAP classes 0, 1, 2, 3 and 4-6 with 3 sera each (set A.1). Another 15 sera were selected with sIgE to HE and CAP classes mentioned above (set A.2). One serum sample was used in set A.1 and A.2. Specific IgE to alpha-lactalbumin, beta-lactoglobulin, ovalbumin and ovomucoid was determined and correlated with semi-quantitative results from ISAC and specific fluorescence from the silicon microarray. One result for alpha-lactalbumin was omitted due to an implausible result. Twelve sera (A.3) with 0 to 0.72 mg_A/l were selected to assess sensitivity to IgG₄ for cow's milk components. These sera included one serum sample also used in set A.1 and two sera used in A.2. Sera of set A.3 were re-assayed in two separate batches. IgE-specific fluorescence was not analyzed in these assays. An additional 12 sera were analyzed for hen's egg specific IgG₄ (set D). IgE-specific fluorescence was not analyzed in these assays. Inter- and intra- slide variation were addressed with a pooled serum (set B). Set C included two sera with high levels of sIgE to CM, HE and components, respectively. These sera were diluted in 14 and 9 steps to at least 0.1 kU_A/l for CM or HE and used in dilution assays. Quantitative measurements in kU_A/l or mg_A/l were performed with the ImmunoCAP 250 platform (Thermo Fisher Scientific, Schwerte, Germany). Results reported-out as <0.35 kU_A/l or 0 mg_A/l were recorded as 0.01. Semi-quantitative results in ISU for set A were obtained by a customized ISAC chip (VBC-GENOMICS, Vienna, Austria; Phadia, Uppsala, Sweden), comparable to version CDR103 and published previously (29). Components measured on the ISAC are referred to with their allergen name by the WHO/IUIS Allergen Nomenclature Sub-Committee. The study was approved by the of the Charité Universitätsmedizin Berlin(EA2/066/11.) and all patients provided written informed consent before entering the study.

Media and Chemicals

Agar, yeast extract, tryptone, NaCl, kanamycin and Isopropyl β-D-1-thiogalactopyranoside (IPTG) were purchased from ROTH (Karlsruhe, Germany). Urea and DTT were from Applichem (Darmstadt, Germany). LDS sample buffer, MES-/MOPS running buffer, antioxidants and pre-cast Bis-Tris mini-gels were from Life Technologies (Carlsbad, USA). Tris-HCl, monosodium phosphate, Tween 20, phosphate-buffered saline (PBS) tablets, N,N-

dimethy-lacrylamide (DMA), and [3-(methacryloyloxy)propyl]trimethoxy-silane (MAPS) and Coomassie Blue were from Sigma (St. Louis, USA). N-Acryloylox-ysuccinimide (NAS) was from Polysciences (Warrington, USA).

Proteins

Native proteins were purchased from Sigma (St. Louis, USA) and had a purity of ≥ 70 to $\leq 98\%$. Egg white powder was provided by OVOBEST Eiprodukte (Neuenkirchen-Vörden, Germany). Lyophilized proteins were diluted in PBS (0.54 to 1.38 mg/ml or 1% for HSA), aliquoted and stored at -20°C until usage, except skim milk protein solution which was freshly prepared at 2 mg/ml, mixed with sodium azide and stored at 4°C . Vectors with cloned sequences of milk and hen's egg proteins were constructed (cDNA accession number in brackets): casein $\alpha 1$ (X00564), casein $\alpha 2$ (NM174528), casein β (XM_010806178), casein κ (X00565), α -lactalbumin (M18780), β -lactoglobulin A (X14712); ovomucoid (XM_015293764.1) and ovalbumin (NM_205152). All inserts were cloned in pET24b(+) vector (Novagen, Madison, WI, USA) with N-terminal T7-Tag sequence and C-terminal His-Tag sequence. HIS-tagged recombinant proteins were expressed in *E. coli* [One Shot BL21(DE3), (Life Technologies, Carlsbad, USA)] and purified by affinity chromatography under denaturing conditions (8 M urea) on mini columns (BioRad, Munich, Germany) according to instructions (The QIAexpressionist, 2006). After dialysis against PBS, protein content was determined by BCA assay (Thermo Scientific, Waltham, USA.). Purity was analyzed by SDS-PAGE and ranged from 69.8 to 97%. Aliquots were stored at -20°C until usage. Concentrations of purified recombinant proteins ranged from 0.49 to 1.75 mg/ml. Monoclonal human IgE kappa from human myeloma serum, purity $>98\%$, was purchased from Nordic Immunology (Tilburg, The Netherlands), and human IgG₄, derived from myeloma cells, purity $>95\%$, from Merck (Darmstadt, Germany).

Antibodies and labels

Streptavidin-Cy3 was purchased from Jackson Immuno Research (West Grove, PA) and from Sigma (St. Louis, USA). Goat anti-human IgE, ϵ -chain specific in serum (#411520), and mouse anti-human IgG₄, Fc Fragment specific (HP6025) (#411492), were purchased from Merck (Darmstadt, Germany). Goat anti-human IgE was labeled with Cy3 and mouse anti-human IgG₄ with Cy5 (GE Amersham, Chalfont St. Giles, UK) *in-house* according to the manufacturer's instructions.

Spotting, assaying and scan settings

The copolymer used to coat the slides, copoly(DMA-MAPS-NAS), was synthesized as described previously (25). Silicon wafers cut into chips of 15x15 mm were obtained from SVM (Sunnyvale, USA). Silicon slides were pre-treated with oxygen plasma for 10 min. After pre-treatment, silicon slides were immersed for 30 min in a copoly(DMA-NAS-MAPS) solution (1% w/v in 0.8 M $(\text{NH}_4)_2\text{SO}_4$ water solution). The slides were then rinsed with water and dried under vacuum at 80°C. Coated chips were stored in a dry chamber and spotted within 15 days.

An array of Streptavidin-Cy3 (reference spots), PBS (negative control), different concentrations of purified IgG₄ and IgE in 1% HSA, 1% HSA and in total 18 allergenic proteins of hen's egg and cow's milk (referred to as analytes) was patterned using an SciFlexArrayer S5 spotter from Scienion (Berlin, Germany) according to the scheme reported in the Supplementary Information (**Supplementary Figure 1**). Native and recombinant proteins were aligned at approximately equal concentrations. Spotting volume was 400 pl, every analyte was spotted in replicates of five. The whole process was performed at room temperature and lasted 3 hours. Chips spotted on one day (usually up to 20) are referred to as one Lot. Chips from this Lot used on the next day or the day after, are referred to as (daily) batch. One batch was comprised of up to 10 chips from which one was reserved for application of the reference pool serum (inter assay control assessment).

After spotting, the chips were stored in a humid chamber overnight to immobilize the proteins onto the surface. The next day, the required amount of chips was blocked with 20 ml blocking buffer (50 mM ethanolamine in 1 M Tris-HCl pH 9) in a large Petri dish in darkness for 1 hour. Each slide was slewed in pure water and dried with nitrogen. Transferred in a separate small Petri dish and placed in a humid chamber, each chip was incubated with 20 µl of undiluted serum in dark at room temperature for 2 hours. After incubation, each chip was slewed in pure water, transferred into a large Petri dish and treated with washing buffer (Tris-HCl 0.05 M pH 9, NaCl 0.25 M, Tween 20 0.05% v/v) for 10 minutes. After brief rinsing with pure water and drying with nitrogen, a mix of 100 µl secondary antibodies (anti-human IgE and anti-human IgG₄) were applied per chip (dilution of antibodies 1:500 in incubation buffer (Tris-HCl 0.05 M pH 7.6, NaCl 0.15 M, Tween 20 0.02% v/v) in darkness for 1 hour. After brief rinsing, washing in a large Petri dish with PBS for 10 minutes and drying with nitrogen, chips were scanned at different laser powers and corresponding PMT gain (photo multiplier). Application settings for spot detection were: Method: footprint, Maximum footprint: 100µm, spot finding algorithm: new fast spot, locating range: 2 pixels, filter data: 3x3 median filter. Quantification settings were: Protocol Easy Quant, quantitation method: adaptive circle with options min. spot diameter (% of nominal): 50, and max. spot diameter (% of nominal):

200, normalization method: LOWESS. Images shown are unadjusted except where mentioned.

Quantification and Statistics

Quantification of IgE(Cy3-channel)- and IgG₄(Cy5-channel)-specific fluorescence was performed with ScanArray Express 4.0 (PerkinElmer). Quantitative and semi-quantitative results, fluorescence, normalized fluorescence, CCU's and normalized CCU's were correlated with Spearman's rank correlation ($r_{(s)}$) and tested two-tailed for significance (SPSS 22.0, IBM). Levels of significance are indicated with asterisks, corresponding to $p < 0.05$ (*) or $p < 0.01$ (**).

IgE-specific signals at 80% laser power and PMT gain were used for quantification of all assays. These signals were combined with IgG₄-specific signals at 60% laser power and PMT gain for sets A, A.1, A.2 and B. Sets A.3, C and D were measured with 70% laser power and PMT gain after replacement of the broken red (Cy5) laser with a weaker one.

With ScanArray software, spot-specific IgE (Channel 1)- and IgG₄ (Channel 2)-specific median, mean, Channel 1 SD, Channel 1 Background SD, Channel 2 SD and Channel 2 Background SD fluorescence and S/N ratios were quantified and transferred to EXCEL (MS OFFICE 2003). In EXCEL, averages and SD of each quantified data for the spotted five replicates per analyte were calculated and adjusted by spot exclusion according to criteria described below.

Processing of raw fluorescence

Smeared, irregular or missing spots were excluded from downstream analyses. For statistical analysis, averaged median and mean fluorescence values were used; background was not subtracted. In addition to visual constraints mentioned above, statistical criteria were also considered. Each spot can be individually assessed by its standard deviation of fluorescence (Ch1 SD), which refers to the homogeneity of the spot, and standard deviation of its surrounding background fluorescence (Ch1 B SD), which is caused by outer spots or noise (lateral fluorescence). As each analyte was spotted in 5 replicates, 5 values for each Ch1 SD and Ch1 B SD were independently ranked. If the highest ranked value exceeded twice the average of the remaining 4 (or less, if visual control had led to exclusion) spots, it was excluded. This procedure was repeated until the highest ranked value was within twice the average of the remaining spots, and applied independently to the IgE and IgG₄ specific fluorescence.

Results

Secondary antibody specificity

Cross-reactivity of secondary antibodies was assessed by mono and co-incubation assays after application of sera with high or low levels of IgE. Fluorescence measured in mono and co-applications of the anti-human IgE-Cy3 and anti-human IgG₄-Cy5 were highly specific, no significant differences were found when total fluorescence of differently incubated slides were compared. This is important for time and cost saving parallel detection of IgE and IgG₄-specific signals in one sample and later calculations of IgE/IgG₄ ratios

Intra- and inter-slide variation

Pooled sera of medium levels of specific IgE (**Table 1**, set B) against hen's egg and cow's milk were used to assess intra- and inter-slide coefficient of variation (CV%). For intra slide variation, 10 chips of one daily batch were incubated and are shown as an example in **Supplementary Figure 2**. Inter-slide variation was calculated from the daily batch controls of all lots, including one randomly chosen chip from the intra-slide run (n=13). For each slide, the mean fluorescence of each of the 5 replicates per analyte (allergens, calibrators, PBS, Blank and HSA) was averaged. Exclusion and clearance criteria as described above were applied. These averages (10 for intra and 13 for inter assay variance) were then used to calculate the %CV for each analyte.

For the mean %CV of intra- and inter-assay variance, recombinant ovalbumin was excluded because it showed no detectable fluorescence for either patients' IgE or IgG₄. Recombinant alpha-lactalbumin was excluded due to poor binding of IgE and IgG₄. It also showed a strong donut-effect and was in part affected by strong fluorescence from native alpha-lactalbumin (spatial interference). Results are shown in **Supplementary Table 1**. In general, CV's for intra-slide variation were lower compared to inter-slide ones. No difference could be detected between median and mean fluorescence. The intra-slide variation for cow's milk allergen IgE-specific mean fluorescence was 11.3% on average, and 26.9% for inter-slide variation. For IgG₄-specific signals, the mean intra-slide variation was 14.1% and the inter-slide variation was 33.1%. Results for hen's egg allergens were very similar with 14.0% (intra-) and 30.0% (inter-) for IgE, and 16.6% (intra-) and 32.2% (inter-) for IgG₄-specific fluorescence.

Sensitivity

Sera in set A with specific IgE to hen's egg and cow's milk were grouped and compared for IgE-specific fluorescence (**Figure 1**). For hen's egg-specific IgE, a clear titer-dependent increase in fluorescence was observed; the microarray assay was sensitive down to the cut-

off of 0.35 kU_A/l. In contrast, results for cow's milk showed no difference between 0.35 and 3.5 kU_A/l. The range of the assay was therefore assessed with another experiment (Linearity of signal/working range).

Calculation and application of different modes of calibration

ImmunoCAP ISAC uses reference material included in the assay kit to provide semi-quantitative results. It would be of great benefit to introduce internal calibrators in the spotted arrays to avoid the use of reference samples run in parallel with assays. We attempted to introduce the spotting and immobilization of known amounts of test analytes (human IgEs and IgGs) to generate an integrated calibration curve and to transform the analyte-specific fluorescence into CCU's (calibration curve units). The procedure is detailed in the Supplementary Information. We also applied normalizations according to intra- and inter-assay variation. Calculated CCU's are listed in **Supplementary Tables 3 and 4**. Correlations of normalized fluorescence are included in **Table 1**.

Correlation with (semi)-quantitative results from ISAC and ImmunoCAP

Quantitative IgE- and IgG₄-specific results from ImmunoCAP (in kU_A/l and mg_A/l, respectively) were correlated with cleared fluorescence derived from the microarray assay, normalized to average intra- and inter- slide variation of fluorescence, units obtained from application of internal calibration (CCU) and a combination of normalization and internal calibration. Results are listed in **Table 2**. Cleared fluorescence and fluorescence normalized to inter-slide variation gave higher positive correlations than other modes of calculation. Despite the high degree of missing CCU, replaced manually with "0" or "4", clearly positive correlations for IgE were observed. IgG₄ specific quantitative results correlated well with processed data. Cleared fluorescence showed very high correlations up to 0.924** (native alpha-lactalbumin), inter normalization of casein-specific fluorescence slightly improved the correlation from 0.884** to 0.896**.

We correlated semi-quantitative results from ISAC (in ISU) with processed fluorescence from our silicon microarray. Most correlations were >0.7 and highly significant.

We also formed clusters of each cow's milk and hen's egg components. The sum of ISU or fluorescences for clustered components of hen's egg and cow's milk proteins was compared with quantitative results for cow's milk and hen's egg from ImmunoCAP. The silicon microarray showed a higher correlation to quantitative results. Native components correlated better compared to recombinant proteins (**Supplementary Table 2**).

Fourteen to 15 sera (sets A.1 and A.2) were selected and specific IgE to components was measured on the ImmunoCAP platform. Results were correlated with fluorescence and

results available from ImmunoCAP ISAC, shown in **Table 2**. The correlation between quantitative alpha-lactalbumin and fluorescence for native alpha-lactalbumin was clearly positive compared to ISAC. Quantitative results for beta-lactoglobulin correlated equally well with ISU and silicon microarray with slight differences depending on the variant of beta-lactoglobulin. We observed more positive correlations with native cow's milk allergens compared to the recombinant variants. The comparison of hen's egg proteins showed an equally good correlation for native ovalbumin on both. Recombinant ovomucoid correlated slightly better compared to the native variant and to the same level as ovomucoid on ISAC. We also chose 12 sera (set D) with specific IgG₄ to hen's egg and found a significant correlation between quantitative IgG₄ and specific fluorescence for hen's egg white (**Table 2**).

Linearity of signal/working range

In order to assess linearity of signal and working range of the assay, we screened two sera (set C) with very high specific IgE against hen's egg or cow's milk and diluted them stepwise 1:2 with 1% BSA in PBS down to ~0.1 kU_A/l, which is the detection limit of the commercial quantitative ImmunoCAP. Results are shown in **Figure 2**.

We observed very clear concentration-dependent fluorescence responses for hen's egg proteins starting at 0.1 kU_A/l for native ovalbumin, native and recombinant ovomucoid. For egg white, it was at least 0.35 kU_A/l. The upper limit for recombinant ovomucoid remained undetected because no saturation of fluorescence occurred, and levels of sIgE to ovomucoid were 60.2 kU_A/l at maximum. Other hen's egg proteins showed an insignificant saturation near levels of 100 kU_A/l. The working range for hen's egg proteins covered all CAP classes. Concentration-dependent responses for cow's milk proteins were at least 0.35 kU_A/l. Stable responses from 1 to 100 kU_A/l were found with skim milk. Native alpha-lactalbumin and native beta-lactoglobulin showed specific responses between 0.1 and 0.35 kU_A/l at the lower end of detection. The upper limit was approximately 30 kU_A/l. Recombinant alpha-lactalbumin and beta-lactoglobulin showed a delayed response from approximately 3.5 kU_A/l to maximal levels of 117 kU_A/l for alpha-lactalbumin and 60.9 kU_A/l for beta-lactoglobulin. Responses for casein strongly fluctuated and were stable from approximately 10 to 75 kU_A/l. We measured specific IgG₄ for alpha-lactalbumin, beta-lactoglobulin, casein, hen's egg, ovomucoid and ovalbumin. As shown in **Figure 3**, the sensitivity for native and in part recombinant alpha-lactalbumin and native beta-lactoglobulin was 0.01 mg_A/l and quickly reached saturation near 1 mg_A/l. Recombinant beta-lactoglobulin and in part recombinant alpha-lactalbumin showed responses between 0.07 and approximately 1.6 mg_A/l. Casein performed quite similar to the proteins mentioned. Hen's egg protein, native and

recombinant ovomucoid showed concentration-dependent fluorescence close to 0.01 mg_A/l IgG₄. The upper limit of the dynamic range could not be properly determined due to an increase of fluorescence in dilution step 5 for all allergens, but it was likely to be near 1 mg_A/l IgG₄ as well.

IgE- and IgG₄-specific responses of casein components are shown in **Supplementary Figure 3**.

Allergen stability/Shelf life

One batch of silicon slides was stored unblocked in a dessicator at 4°C. On days 1, 9, 40 and 60 a slide was incubated with pooled sera (set B) and IgE-specific fluorescence was determined. Stable responses were observed for all allergens up to day 40. After 60 days, fluorescence of most allergens dropped significantly.

Discussion

In this paper we described the development of a polymer coated silicon microarray for simultaneous detection of food allergen-specific IgE and IgG₄ which overall showed good correlation to quantitative and semi-quantitative results from the ImmunoCAP and ImmunoCAP ISAC platforms. We will discuss here the strengths and limitation of our system.

Auto fluorescence and secondary antibody specificity

Auto-fluorescence of spotted proteins was not detected. Low to moderate background fluorescence was detected on IgE-specific Cy3-channel and attributed to non-specific binding of the secondary polyclonal antibody along with physicochemical characteristics of the Cy3-chromophor. On Cy5-channel, background was negligible. In general, native and recombinant kappa-casein showed a high background on both channels. However, for correlation coefficients, we did not apply any subtraction of baseline fluorescence or background fluorescence. Tests of changes of laser power and corresponding PMT gain did not reveal combinations more suitable than those applied.

Intra- and inter-slide variation

In general, the IgE- and IgG₄-specific intra-slide variations of the mean fluorescence were good with 12.6% and 15.7% on averages, respectively. Results of inter-slide variation were higher with 30.7% for IgE and 32.9% for IgG₄. The silicon microarray performed considerably

better in comparison to available results for components of inhaled allergens on ISAC. In a study by Meliolo, Bonifazi et al. (30), intra-assay CV's on ISAC in the low range of 0.3 to 1 ISU were greater than 100%, and CV's for ISU between 1 to 15 were determined with 17% (intra-assay) and 33% (inter-assay). In our study, a pool of sera (set B) was used as intra- and inter- assay controls. It was assayed on ISAC and 0.68 ISU for alpha-lactalbumin and 0.54 ISU for beta-lactoglobulin were determined. CV's on the silicon microarray for IgE-specific mean fluorescence of native alpha-lactalbumin was 6.8% (intra-assay) and 16.2% (inter-assay). For native beta-lactoglobulin, 16.2 and 20.2% were calculated. In the range of 1 to 15 ISU, ovomucoid and ovalbumin were analyzed on ISAC and found to be 3.67 and 1.28 ISUs. CV's for native ovomucoid and ovalbumin on the silicon microarray were 19.6% and 11.1% for intra-assay, and 26.1% and 24.8% for inter-assay variance. IgG₄-specific CV's are not directly comparable, because we did not determine sIgG₄ on ISAC. Details are shown in the **Supplement Table 2**. These results are strong evidence of higher reproducibility of the silicon microarray and confirm previous findings by Cretich, Di Carlo et al. (25).

This relatively high inter-slide variation can be ascribed to several factors: most steps in assaying the slides were manual (e.g. washing), several batches and lots of slides were used, only one array per slide was spotted (ISAC has 4 arrays per slide), the slide was scanned in a holder that was not completely level. Apart from these factors that could quite easily be solved, a well-known problem is the spotting procedure itself with an estimated error of 20-25% in depositing protein onto the surface (31). This would imply using 3 colors for an additional total protein control, which would provide further data on the quality of spotting, but would also increase costs or require the introduction of an internal calibration method to normalize the allergen fluorescence response by its actual immobilization yield (32). Poor binding of antibodies to proteins, e.g. recombinant alpha-lactalbumin, may also provoke relatively high CV's by cumulative effects of low positive fluorescence and high standard deviation resulting from differences in protein deposition from chip to chip. Another well known problem that affects proper quantification and thus inter-assay variability is sub-optimal spot morphology ("donut-effect"). The ring-shaped structure was most notable for beta-casein. According to Wellhausen and Seitz (33), this effect could be compensated for by application of e.g. Triton or glycerol, which lower the surface tension of the protein solution, or mathematically by using median instead of mean fluorescence. In this study, both mean and median fluorescence were correlated with quantitative results and showed very similar coefficients.

Sensitivity and correlation with other platforms

We applied several normalization steps to the fluorescent data in order to determine the (limited) benefit of processing. Highest correlations to quantitative and semi-quantitative data were calculated for cleared fluorescence and inter-slide normalized fluorescence, which was therefore considered most suitable for downstream correlations.

Hen's egg-specific IgE measured on our polymer coated silicon microarray correlated well with the ImmunoCAP system and showed sensitivity in the low range down to $<0.35 \text{ kU}_A/\text{l}$. In contrast, for cow's milk-specific IgE, only limited sensitivity and correlation could be found. In this case the assay did not show sufficient sensitivity to clearly distinguish quantitatively determined negative from positive sera up to $3.5 \text{ kU}_A/\text{l}$. It became clear that the assay has limits in precise detection at low and very high concentrations. As shown by Gaudin et al. (34), large differences between cow's milk substrates exist. The high number of allergenic components, (4 caseins, alpha-lactalbumin, beta-lactoglobulin and other allergens not assessed with our assay) which differ in content depending upon how the cow's milk is processed, and matrix-dependent binding affinities contribute to divergent performances on different platforms. Therefore, skim milk might be an inappropriate analyte in comparison to the cow's milk panel on the ImmunoCAP.

IgG₄-specific correlations for native components were highly positive with $r_{(s)}$ between 0.849** and 0.924**. Sensitivity of the silicon microarray in the range 0.00 to $0.72 \text{ mg}_A/\text{l}$ was assessed with 12 sera determined for total hen's egg, alpha-lactalbumin, beta-lactoglobulin and casein specific IgG₄. Correlations for native alpha-lactalbumin and beta-lactoglobulin were similar to those obtained from 91 sera. Casein correlated positive, but failed to be significant. Recombinant alpha-lactalbumin correlated negatively which is confirmed by the observation of less high affinity to IgE compared to native alpha-lactalbumin (**Figure 3**). According to Thermo Fisher, the LOD for IgG₄ on the ImmunoCAP platform is $0.07 \text{ mg}_A/\text{l}$, but values down to $0 \text{ mg}_A/\text{l}$ are reported. Therefore correlations of fluorescence with values below the LOD are questionable, and the sensitivity of the copoly(DMA-NAS-MAPS) Si/SiO₂ chip is hard to determine due to missing reliable quantitative references in this range and "real" negative controls. We ascribe this to the general problem of measuring IgG₄ in blood samples. Due to its higher abundance compared to IgE, serum samples are diluted 1:50 (ISAC) or even 1:100 (CAP) prior to assaying. This difference in concentration may compensate for the different binding kinetics of IgE and IgG₄ towards shared epitopes. If this was the case, the assay would mimic physiological conditions in the patient with implications for the role of IgG₄ as competing with IgE.

Despite these significant correlations, considering the CVs and correlations calculated from the 91 sera, these results also verified the finding that some components, e.g. caseins, are difficult to measure accurately.

As shown in assays with geometric dilutions of two sera in 1% BSA in PBS, the parallel measurement of IgE and IgG₄-specific fluorescence in one assay is possible, although the working range for IgG₄ seems to be limited from 0.01 to 1 mg_A/l, whereas the ImmunoCAP's range is up to 30 mg_A/l. We observed a higher affinity of IgG₄ to native alpha-lactalbumin and beta-lactoglobulin compared to recombinant variants in our assay. It is likely that the tertiary structure has not been maintained exactly and thus some conformational epitopes would be lost.

The results for IgE levels in these assays showed a very good performance for hen's egg proteins over the clinical relevant range from approximately 100 kU_A/l to 0.1 kU_A/l with the exception of recombinant ovalbumin. On the other hand, recombinant ovomucoid exceeded its native variant in sensitivity and working range. This finding may result from different spot morphology or the fact that recombinant ovomucoid is not glycosylated and binding sites are easily accessible. Results of cow's milk specific IgE and dilutions revealed that the sensitivity for skim milk is about 1 kU_A/l with clear titer-dependent increase of fluorescence up to 100 kU_A/l. Cow's milk components' fluorescence also correlated with IgE titer, however, saturation levels differ.

It became clear that food allergens are challenging to assay reliably on microarrays and that component specific cut-offs or adjusted protein content (e.g. kappa casein) are likely to be necessary. Calibration and normalization, close monitoring of spot quality and protein content, and clearly defined quality criteria and data processing are indispensable. In order to clearly compare different platforms, repeated measurements with standard materials (serum and allergens) under defined conditions need to be performed side-by-side (24, 35).

Further analysis of the data with clinical symptoms are necessary to determine IgE-specific allergen-specific cut-offs. The reason for failure of some recombinant proteins (especially ovalbumin and in part alpha-lactalbumin) is yet unknown, tests of function in e.g. Western blots will be applied. Shelf life may be prolonged by optimization of storage conditions.

In summary, we showed that the copoly(DMA-NAS-MAPS) Si/SiO₂ based microarray is a semi-quantitative assay with a working range comparable to the ImmunoCAP ISAC. Correlations between quantitative results from the ImmunoCAP correlated better with IgE-specific fluorescence from the silicon based microarray than with ISUs from ISAC. There is evidence that copoly(DMA-NAS-MAPS) Si/SiO₂ array is more sensitive in IgE detection than the ImmunoCAP ISAC, as shown in geometric dilution assays. Parallel measurements of IgE and IgG₄ are possible, which could be useful for the diagnosis of food allergy, because IgG₄ has been suggested as a candidate involved in tolerance development (36-38). Therefore, quantitative data are needed, preferably simultaneously from the same blood sample in low

volume (39, 40). A *quantitative* microarray platform in routine diagnostics would fulfill these needs.

The lack of quantitative results and sensitivity, the fixed set of allergens, the high costs and the limitation due to missing possibilities to detect IgE and IgG₄ simultaneously complicate the acceptance of the ImmunoCAP ISAC. On the other hand, considerable improvements were incorporated from version CDR103 to the CDR112 (41-43). In allergy research the ISAC has helped support the idea of molecular spreading, which has given insight into the relationships between the sequential occurrence of specific IgE to inhalant allergens and prognosis of later clinical reactions (44). For food allergy, no initiating molecule has so far been identified, and the concept of molecular spreading has not been proven yet.

We have previously demonstrated that microfluidics coupled to microarrays can improve the accuracy of IgE vs IgG detection when low and high affinity antibodies compete for the binding with the same allergen (45). This might be another advantage of the silicon microarray, beneficial for diagnostics. Slides with a four-field-format, together with the known relatively cheap and easy-to-perform surface chemistry, might make the copoly(DMA-NAS-MAPS) silicon chip an accessible and low cost alternative for use in research labs compared to the commercial ImmunoCAP ISAC.

Figure legends

Figure 1: Correlation of (A) hen's egg and (B) cow's milk specific IgE, determined on ImmunoCAP, and IgE specific mean fluorescence of egg white and skim milk measured with the copoly(DMA-NAS-MAPS) silicon array. Calculated correlation coefficients are 0.814** and 0.749**. The dashed lines indicate CAP classes on ImmunoCAP.

Figure 2: IgE-specific mean fluorescence at 80% laser power and PMT gain for cow's milk (A1, B1), hen's egg (A2, B2) and respective components from two sera (set C). The dashed line indicates the 0.35 kU_A/l cut-off on ImmunoCAP. The limit on the abscissa was set to 0.001 and to 10 on the ordinate.

Figure 3: IgG₄-specific mean fluorescence at 70% laser power and PMT gain for cow's milk (A1, B1), hen's egg (A2, B2) and respective components from two sera (set C). The dashed line indicates LOD of 0.07 mg_A/l on ImmunoCAP. The limit on the abscissa was set to 0.001 and to 10 on the ordinate.

Tables

Table1: Sets of sera used in this study. Quantitative and semi-quantitative data are presented for sets B and C in absolute values and for sets A, A.1, A.2 and D as median and range (minimum – maximum, in brackets) Allergen names according to the World Health Organization and International Union of Immunological Societies (WHO/IUIS) are given in brackets. Abbreviations: n/a, not available on platform; n.d., not determined; n.u., not used, data available, but not evaluated

Set	n	parameter	ImmunoCAP		ISAC	
			slgE in kU _A /l median (range)	slgG ₄ in mg _A /l median (range)	parameter	slgE in ISU
A	91	hen's egg	0.8 (0.01 – 102)	n.d.	ovomucoid (nGal d1)	0 (0 - 58.21)
					ovalbumin (nGal d2)	0 (0 - 18.30)
		cow's milk	2.71 (0.01 – 365)	n/a	αs1-casein (Bos d8αs1)	0 (0 - 30.05)
					β-casein (Bos d8β)	0 (0 - 14.25)
		α-lactalbumin	n.d. in general	0.2 (0 - 14.2)	κ-casein (Bos d8κ)	0 (0 - 33.56)
		β-lactoglobulin	n.d. in general	0.12 (0 - 23.6)	casein (Bos d8)	0 (0 - 26.23)
		casein	n.d. in general	0.48 (0 – 276)	α-lactalbumin (Bos d4)	0 (0 - 15.72)
A.1	14	α-lactalbumin	0.21 (0 – 34)	n.u.	β-lactoglobulin (Bos d5.0101)	0 (0 - 15.21)
	15	β-lactoglobulin	1.04 (0.01 - 8.87)	n.u.	β-lactoglobulin (Bos d5.0102)	0 (0 - 9.57)
						0 (0 - 5.96)
A.2	15	ovomucoid	1.06 (0 - 29.4)	n.u.	ovomucoid (nGal d1)	1.48 (0 - 28.45)
		ovalbumin	0.55 (0 – 59)	n.u.	ovalbumin (nGal d2)	1.86 (0 - 14.95)
A.3	12	α-lactalbumin	n.u.	0.045 (0 – 0.72)	α-lactalbumin (Bos d4)	n.d.
		β-lactoglobulin	n.u.	0.085 (0 – 0.42)	β-lactoglobulin (Bos d5.0101/02)	n.d.
		casein	n.u.	0.045 (0 – 0.70)	casein (Bos d8)	n.d.
B	1 (pool)	hen's egg	18.9	n.d.	hen's egg	n/a
		ovomucoid	26.3	n.d.	ovomucoid (nGal d1)	3.67
		ovalbumin	25.7	n.d.	ovalbumin (nGal d2)	1.28
		cow's milk	12.0	n/a	cow's milk	n/a
		α-lactalbumin	5.47	n.d.	α-lactalbumin (nBos d4)	0.68
		β-lactoglobulin	4.06	n.d.	β-lactoglobulin (nBos d5)	0.54
		casein	11.7	n.d.	casein (nBos d8)	3.28
C	2	hen's egg	56.8 ; 98.4	0.75 ; 1.12	hen's egg	n/a

D	12	ovomucoid	47.6 ; 60.20	0.39 ; 1.29	ovomucoid (nGal d1)	n.d.
		ovalbumin	42.0 ; 62.40	0.15 ; 2.08	ovalbumin (nGal d2)	n.d.
		cow's milk	727 ; 6.60	n/a	cow's milk	n/a
		α -lactalbumin	117 ; 0.51	7.99 ; 0.38	α -lactalbumin (nBos d4)	n.d.
		β -lactoglobulin	60.9 ; 0.92	4.87 ; 2.41	β -lactoglobulin (Bos d5.0101/02)	n.d.
		casein	310 ; 6.17	8.17 ; 3.15	casein (nBos d8)	n.d.
		hen's egg	n.u.	0.09 (0 - 0.67)	hen's egg	n.a.

Table 2: Correlations (Spearman's Correlation Coefficient, two-tailed) of (semi)quantitative results for chosen allergens (left column) with differently processed results from fluorescent detection (mean fluorescence) of spotted allergens (right column) for n=91 sera. FI=fluorescence (cleared), CCU=Calibration Curve Units, FI intra norm=fluorescence (cleared) normalized to averaged intra assay values, FI inter norm= fluorescence (cleared) normalized to averaged inter assay values; CCU intra norm= Calibration Curve Units normalized to averaged intra assay variation, CCU inter norm= Calibration Curve Units normalized to averaged inter assay variation.

n=91	(semi)quantified analyte [unit]	FI	FI intra norm	FI inter norm	CCU	CCU intra norm	CCU inter norm	spotted analyte
IgE specific results	Hen's egg [kU _A /l]	0.814**	0.736**	0.736**	0.705**	0.680**	0.678**	egg white
	Cow's milk [kU _A /l]	0.749**	0.735**	0.735**	0.765**	0.769**	0.761**	skim milk
	Bos d4 [ISU]	0.758**	0.745**	0.778**	0.745**	0.779**	0.790**	nat α-lactalbumin
		0.602**	0.503**	0.575**	0.503**	0.517**	0.472**	rec α-lactalbumin
	Bos d5.0102 [ISU]	0.778**	0.770**	0.855**	0.770**	0.705**	0.688**	nat β-lactoglobulin (A + B)
		0.718**	0.729**	0.712**	0.729**	0.641**	0.590**	rec β-lactoglobulin (A)
	Bos d5.0101 [ISU]	0.681**	0.658**	0.762**	0.658**	0.587**	0.580**	nat β-lactoglobulin (A + B)
		0.689**	0.726**	0.766**	0.726**	0.698**	0.624**	rec β-lactoglobulin (A)
	Bos d8 [ISU]	0.747**	0.724**	0.733**	0.724**	0.702**	0.705**	total casein
	Bos d8as1 [ISU]	0.731**	0.717**	0.764**	0.717**	0.744**	0.757**	rec αs1-casein
	Bos d8bcas [ISU]	0.721**	0.671**	0.759**	0.671**	0.626**	0.702**	nat β-casein
		0.613**	0.583**	0.700**	0.583**	0.570**	0.644**	rec β-casein
	Bos d8kcas [ISU]	0.720**	0.697**	0.682**	0.697**	0.685**	0.684**	nat κ-casein
		0.692**	0.670**	0.782**	0.670**	0.749**	0.784**	rec κ-casein
	Gal d1 [ISU]	0.805**	0.798**	0.828**	0.798**	0.811**	0.804**	nat ovomucoid
		0.804**	0.805**	0.846**	0.805**	0.757**	0.797**	rec ovomucoid
	Gal d2 [ISU]	0.715**	0.731**	0.667**	0.731**	0.628**	0.592**	nat ovalbumin
		0.241*	0.079	0.035	0.079	-0.152	-0.135	rec ovalbumin
IgG4 specific results	α-lactalbumin (Bos d4) [mg _A /l]	0.916**	0.912**	0.895**	0.912**	0.914**	0.912**	nat α-lactalbumin
		0.691**	0.667**	0.662**	0.667**	0.681**	0.663**	rec α-lactalbumin
	β-lactoglobulin (Bos d5) [mg _A /l]	0.843**	0.814**	0.816**	0.814**	0.805**	0.808**	nat β-lactoglobulin (A + B)
		0.841**	0.767**	0.818**	0.767**	0.760**	0.736**	rec β-lactoglobulin (A)

casein (Bos d8) [mg _A /l]	0.884**	0.869**	0.896**	0.869**	0.888**	0.886**	total casein
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Table 3: Correlations of quantitative results (IgE) for CM and HE components with ImmunoCAP ISAC (ISU) and Copoly(DMA-NAS-MAPS) silicon microarray (mean fluorescence). ¹⁾ refers to the sum of Bos d5.0102 (β -lactoglobulin A) and Bos d5.0101 (β -lactoglobulin B); ²⁾ refers to Bos d5.0102 (β -lactoglobulin A). Correlations of quantitative results for IgG₄ in the range 0 to 0.72 mg_A/l were also calculated for cow's milk components.

set	ImmunoCAP	ISAC	Silicon Microarray	spotted analyte
A.1	α -lactalbumin (IgE)	0.781**	0.957**	nat α -lactalbumin
			0.625*	rec α -lactalbumin
	β -lactoglobulin (IgE)	0.831** ¹⁾	0.815**	nat β -lactoglobulin (A+B)
		0.704** ²⁾	0.667**	rec β -lactoglobulin (A)
A.2	ovomucoid (IgE)	0.932**	0.896**	nat ovomucoid
			0.928**	rec ovomucoid
	ovalbumin (IgE)	0.906**	0.872**	nat ovalbumin
			0.057	rec ovalbumin
A.3	α -lactalbumin (IgG ₄)	n/a	0.857**	nat α -lactalbumin
		n/a	-0.242	rec α -lactalbumin
	β -lactoglobulin (IgG ₄)	n/a	0.791**	nat β -lactoglobulin (A+B)
	total casein (IgG ₄)	n/a	0.530	total casein
D	hen's egg (IgG ₄)	n/a	0.691*	egg white

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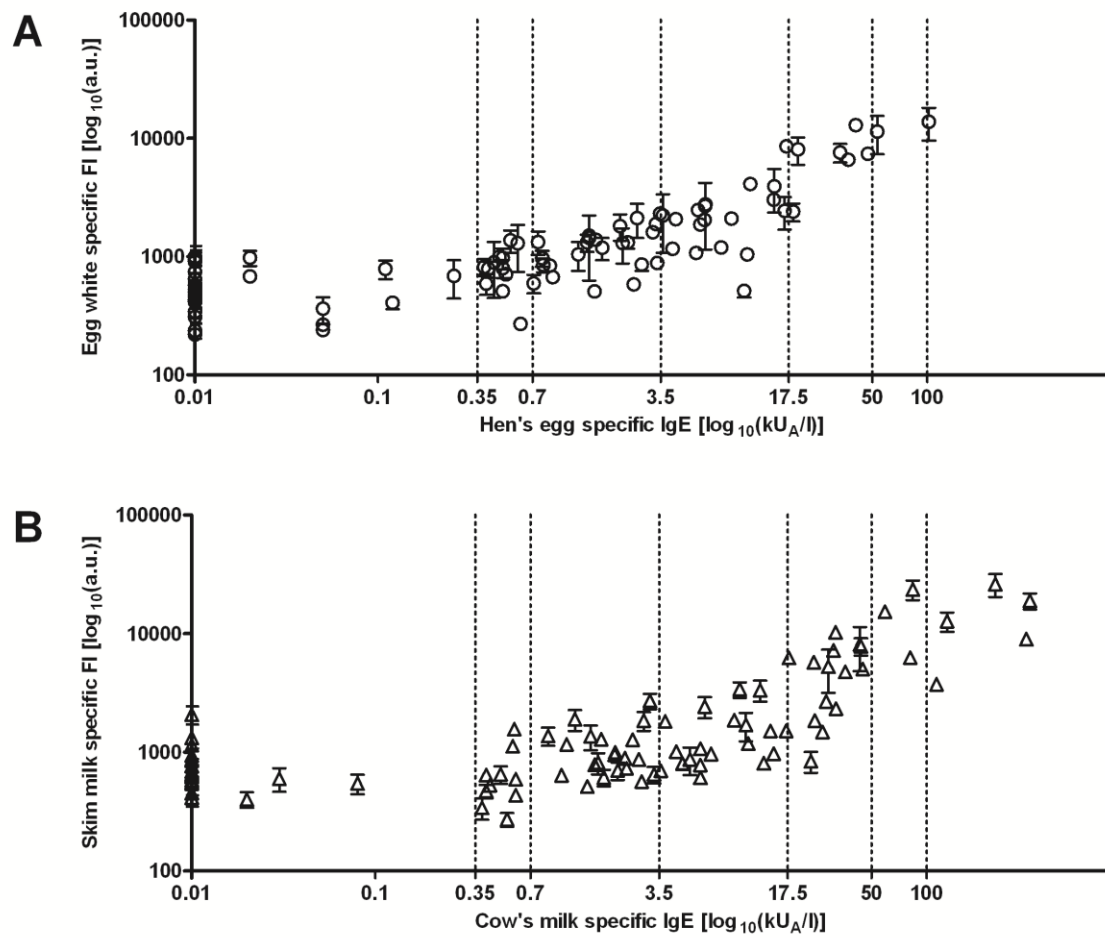
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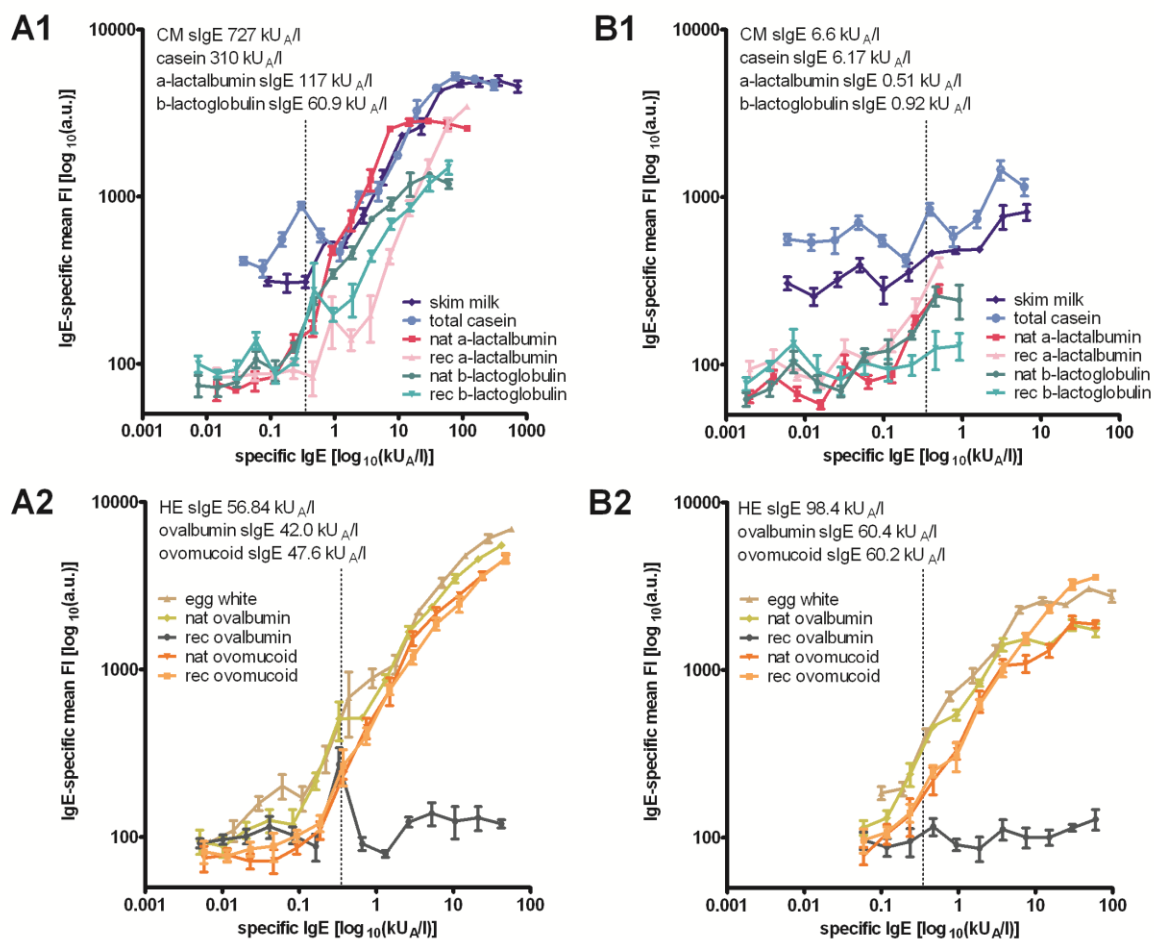
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Author contribution

Steven Sievers, Marcella Chiari, Marina Cretich and Kirsten Beyer were involved in the concept and design of the study and interpretation of the data. Steven Sievers, Paola Gagni and Marina Cretich were involved in the acquisition of the laboratory, Kirsten Beyer, Birgit Ahrens and Bodo Niggemann of the clinical data. Hugh Sampson and Galina Grishina were involved in the provision of the vectors. Steven Sievers purified the recombinant proteins and wrote the first draft of the manuscript. All authors participated in the revision of the manuscript and gave their final approval.



Geometric dilutions of two sera and concentration-dependent IgE-specific fluorescence of extracts and components



Geometric dilutions of two sera and concentration-dependent IgG₄-specific fluorescence of extracts and components

