

Letter to the Editor

Linear epitope mapping of peanut allergens demonstrates individualized and persistent antibody-binding patterns

To the Editor:

Peanut allergy is considered a major health problem due to its prevalence, persistence, and association with severe symptoms.¹ Previous studies suggest that clinical reactivity to allergens might be related to allergen-specific IgE epitope patterns, diversity, and avidity, all of which likely play a role in the effect of IgE on basophils and mast cells.^{2,3} Previous peptide microarray-based studies have demonstrated a pronounced heterogeneity in the epitope-binding patterns between individual allergic patients apart from a few immunodominant epitopes.^{2,4-7} Furthermore, elevated IgG₄ levels have been associated with protective effects.⁸

Here, high-density peptide arrays were applied to map linear IgE and IgG₄ epitopes at the single amino acid level. The peptide arrays contained tiled arrays of 12-mer peptides covering the peanut allergens Ara h 1, 2, 3, 6, 8, and 9. Corresponding peptides with alanine substitutions were used to obtain single amino acid resolution of epitopes and their immune reactivity. Maps of IgE and IgG₄ reactivity were obtained for 5 control subjects and 6 severely allergic patients (ages 8-40 years) sampled across 4 to 10 years (see Table E1 and the Methods section in this article's Online Repository at www.jacionline.org).

Sera from peanut-allergic patients (P1-P6) had extensive IgE and IgG₄ reactivity against peanut allergens compared with sera from control subjects (see Fig E1 in this article's Online Repository at www.jacionline.org). Reactivity was mostly toward Ara h 1, 2, and 3 (see Figs E2-E4 in this article's Online Repository at www.jacionline.org), which corroborates previous studies.^{2,5} Corresponding results using alanine substitutions are shown in Fig 1 and Figs E5-E7 in this article's Online Repository at www.jacionline.org and provide detailed information about the reactivity to the respective epitopes. IgE and IgG₄ binding patterns were heterogeneous between patients but very similar for each patient over time. Some regions, for example, region 85-95 of Ara h 1, were reactive to all investigated patient sera, whereas other regions, for example, 285-300 of Ara h 1, were reactive to some but not all patient sera. The majority of these prevalent regions overlapped with previously described prevalent regions (detailed in Table E2 in this article's Online Repository at www.jacionline.org).

A detailed inspection of the epitopes revealed that certain amino acids (eg, 97P) were important for reactivity in some but not all patients, whereas other amino acids (eg, 99D) were critical for antibody binding in all patients (see Fig 2 and Fig E8 in this article's Online Repository at www.jacionline.org). The observed microheterogeneity in reactivity between patients toward some epitopes may have an impact on the design of epitopes for peptidic allergy vaccines.

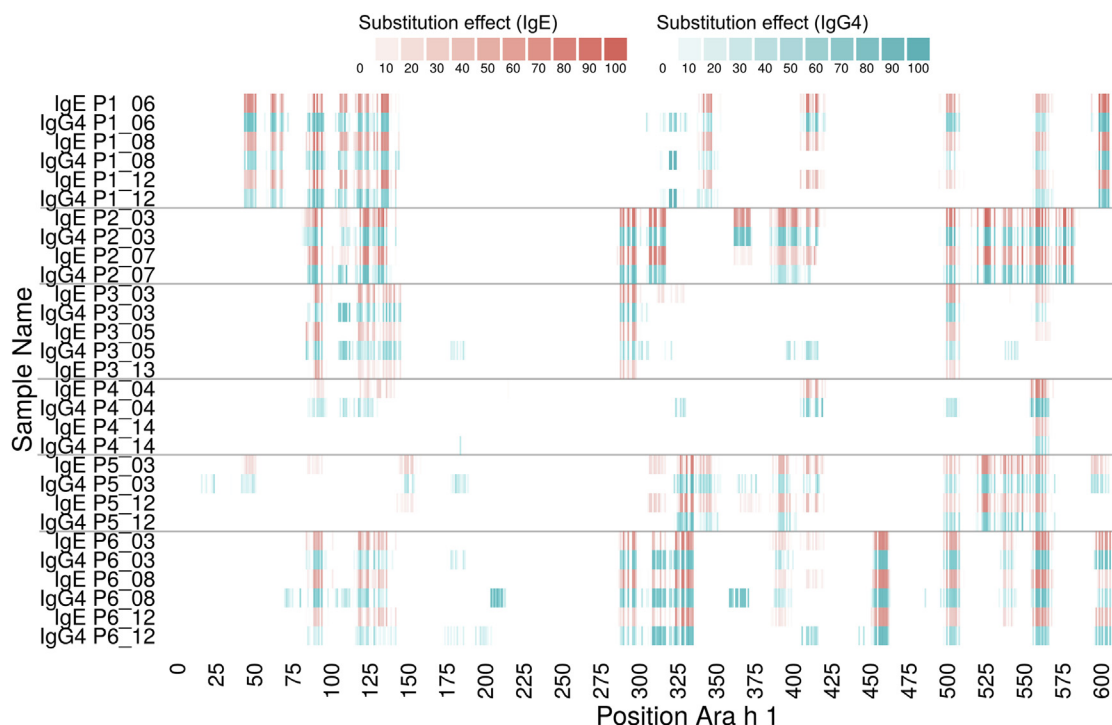


FIG 1. Effect of alanine substitution in Ara h 1 on antibody binding. The relative effect of substituting a given residue (x-axis) to an alanine is reported as 0% to 100% loss of signal compared with the signal of the native 12-mer peptide; that is, a high substitution effect signifies that the residue is important for antibody binding. Each row indicates IgE or IgG₄ signal from patient samples P1 through P6 at a certain year of sampling (eg, P1_06 is patient 1 sampled in 2006).

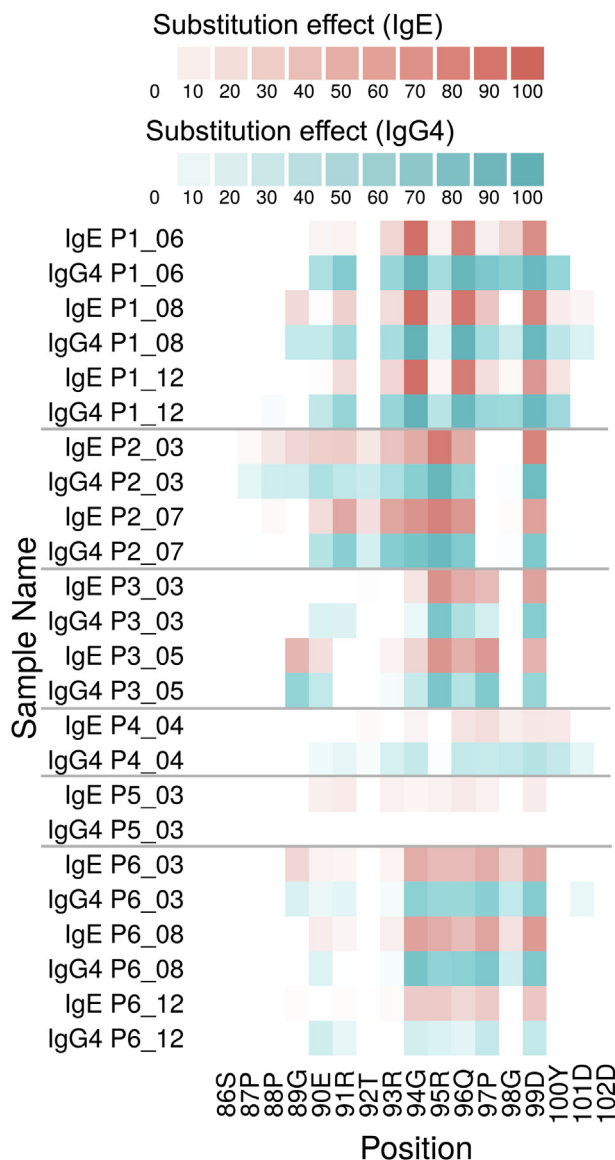


FIG 2. Alanine substitution effect for a sample epitope in Ara h 1 is depicted at high resolution. See legend of Fig 1 for additional details.

The IgE and IgG₄ epitope regions very often colocalized. On average, 68.6% of the reactive peptides bound both IgE and IgG₄. Notably, there was no cross-reactivity between the anti-IgE and anti-IgG₄ secondary antibodies as indicated by ELISA tests (data not shown). At the single amino acid level, the binding patterns were also very similar for IgE and IgG₄ antibodies (Figs 1 and 2 and Figs E5-E7). This may indicate that the IgE and IgG₄ antibodies are clonally related. The findings indicate that specific IgG₄ is regularly present in the allergic response toward peanuts.

The immune-reactivity patterns were remarkably stable over a long period of up to 10 years (see Fig E9 in this article's Online Repository at www.jacionline.org). Spearman rank correlation of the substitution effects for samples obtained from the same patient was higher (inpatient variability, average 0.85 for IgE and 0.78 for IgG₄) compared with the corresponding correlation between different patients (interpatient variability, average 0.52

for IgE and 0.48 for IgG₄). These findings indicate long-term clonal persistence of allergen-specific antibody-producing cells, with potential implications for peanut immunotherapy.

In summary, the use of a new generation of peptide microarrays made the description of linear epitope patterns at a single amino acid level possible. The method is scalable to large patient cohorts and can be used as a standardized tool to follow the immune response in great detail.

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Supplementary information – “Linear epitope mapping of peanut allergens demonstrates individualized and persistent antibody binding patterns”

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Methods

Study population

Patients with peanut allergy were recruited at the Department of Pediatrics, Medical University of Vienna, Austria. In total, 17 samples obtained at different time points from 7 peanut allergic patients (P1-P7) were analyzed. This corresponded to 2 or 3 samples from each patient, which covered an average time interval of 8.3 years (range 4-10 years). In addition, sera from 5 subjects (C1-5) non-sensitized to peanuts were included as controls. No peanut-specific IgE reactivity was observed for the control samples. For unknown reasons, the P7 sera did not show any binding to the peptide array and was therefore omitted from further analyses. One sample from P3 (P3_13) had unspecific IgG4 signal and was likewise omitted from further analyses. The peanut allergic patients were diagnosed by an experienced allergologist and had a convincing history of severe peanut-related symptoms. The clinical history for each patient can be found in Table E1 below. The protocol was approved by the local Ethical Committee of the Medical University of Vienna and written informed consent was obtained (protocol no. EK 428/2008).

High-density peptide microarrays

A peptide library consisting of 4,173 single-offset overlapping 12-mer peptides covering the primary sequence of Ara h 1, 2, 3, 6, 8 and 9 in triplicates was generated (i.e. each peptide was synthesized on three independent spots for each array). Furthermore, the library consisted of 17,777 12-mer peptides corresponding to the non-redundant set of all single-residue alanine derivatives of the native peptides yielding a total allergen peptide library of 22,490 peptides. Serving as control peptides, the library included 50,582 peptides deriving from peanut-unrelated proteins, specifically 148 snake venom toxins. Peptide synthesis was accomplished as described previously (Christiansen *et al.*, Sci. Rep. 5, 12913, 2015) by *in-situ* photolithographic synthesis in a Roche-NimbleGen Maskless Array Synthesizer using an amino functionalized substrate carrying a photosensitive protection group.

IgE and IgG4 Immunoassay

Samples were diluted using 1:2 dilution (for serum) or 1:4 dilution (for plasma) in binding buffer (0.01M Tris-Cl, pH 7.4, 1% alkali-soluble casein, 0.05% Tween-20) and bound to arrays overnight at 4°C. The arrays were washed 3x in wash buffer (1x TBS, 0.05% Tween-20), 10 min per wash. This was followed by incubation with goat anti-human IgE conjugated with tetramethylrhodamine (Thermo Fisher Scientific, A18798) and mouse anti-human IgG4 conjugated with Alexa Fluor® 647 (SouthernBiotech, 9200-31). The secondary antibody was diluted 1:1000 in secondary binding buffer

(1x TBS, 1% alkali-soluble casein, 0.05% Tween-20). Arrays were incubated with secondary antibody for 3 h at room temperature, then washed 3x in wash buffer (10 min per wash) and 30 sec in reagent-grade water. Fluorescent signal of the secondary antibody was detected by scanning at 635nm (IgG4) and 532 (IgE) at a 2µm resolution and 25% gain, using an MS200 microarray scanner (Roche NimbleGen). Raw fluorescent signal intensities were extracted using NimbleGen DEVA signal extraction software.

Analysis - Signal filtration and smoothing

The fluorescence intensity of all recorded peptide fields was mapped back on the originating peanut allergen and indexed as the position of the first peptide residue in the allergen. To filter away potential microarray artefacts, the median intensity was used for all triplicate signals. Next, a rolling median of the peptides with a window size of 3 was applied (i.e. the median signal of a given peptide and the two adjacent peptides); effectively eliminating singular peptide responses. A normalization step was implemented in order to compare signals between different microarray reactions. First, all peptide signals were normalized according to the baseline signal from the non-relevant peptides derived from snake toxins (see above). This was accomplished for each well and antibody subtype by iteratively calculating the baseline mean of the non-relevant peptides, removing outliers more than 2 standard deviations away until a constant mean, m , was obtained. Next, a standard deviation, s , was calculated on all non-relevant peptides with a signal lower than the baseline mean. Finally, a signal-to-baseline Z-score was calculated for each peanut peptide signal, x , using $Z = (x - m)/s$. To correct for peptides consistently showing a signal above baseline, the Z-score was subtracted by the maximum Z-score of identical peptides probed with control serum. The control sera were from subjects with no detected IgE reactivity and no epitopes (peaks) were observed in the raw data. The process of normalization and correction is illustrated on actual data in Figure E2 in below.

A cutoff of two standard deviations ($Z > 2$) was used to define positive IgE and IgG4 binding peptides. This cutoff was used for i) calculation of percentage of allergen sequence with positive binding peptides, ii) percentage of patients sharing positive binding peptides and iii) for determining which peptides should be included in the alanine-substitution analysis.

Alanine-substitution analysis

A complete alanine scan of all native peanut-originating peptides was included on the microarray. Each substituted peptide was expressed as the percentage loss of signal intensity relative to the corresponding native peptide. To report the substitution effect, the median percentage loss in signal of overlapping peptides substituted within the same allergen residue was calculated. Only substitutions in positive native peptides (see above) were included in the median calculation. For

comparison of the substitution effect of epitope binding residues between samples, a Spearman rank-order correlation coefficient was calculated on the median substitution effect of residues from one sample, with those of another, given the value was present in both samples.

Patient ID	Sample ID	Age at sampling	Gender	Symptoms
P1	P1_06	18	f	Anaphylaxis; bronchial obstruction, drop blood pressure, angioedema, oral edema, eczema, urticaria, gastro-intestinal symptoms
	P1_08	22		
	P1_12	26		
P2	P2_03	8	f	Anaphylaxis, bronchial obstruction, urticaria
	P2_07	12		
P3	P3_03	26	m	Anaphylaxis, drop of blood pressure; gastro-intestinal symptoms
	P3_05	28		
	P3_13	36		
P4	P4_04	30	f	Angioedema, gastro-intestinal symptoms
	P4_14	40		
P5	P5_03	22	m	Breathlessness, bronchial obstruction, gastrointestinal symptoms
	P5_12	31		
P6	P6_03	25	m	Gastro-intestinal symptoms, oral edema, bronchial obstruction
	P6_08	30		
	P6_12	34		
	P7_14	41		
P7	P7_06	33	m	Angioedema, urticaria
	P7_14	41		

Table E1: Patient sample overview and clinical symptoms.

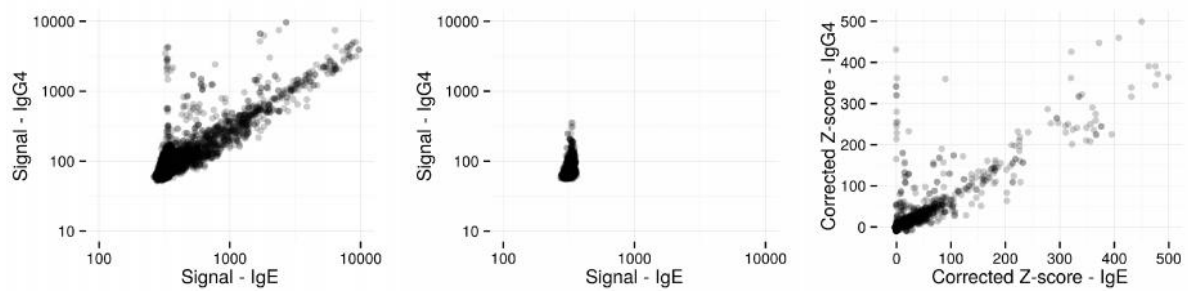


Figure E1: Scatterplots of IgE versus IgG4 signal. **(A)** Signal from peanut-peptides probed with peanut allergic patient samples (N = 25,452). **(B)** Signal from peanut-peptides probed with control samples with no peanut reactivity (N = 9,090). **(C)** Like (A) but depicting the corrected Z-score value for each peanut-peptide. Outlier filtration has been applied on triplicate peptides, see Methods for details. Effects of normalization and Z-scoring are described in detail in figure E10.

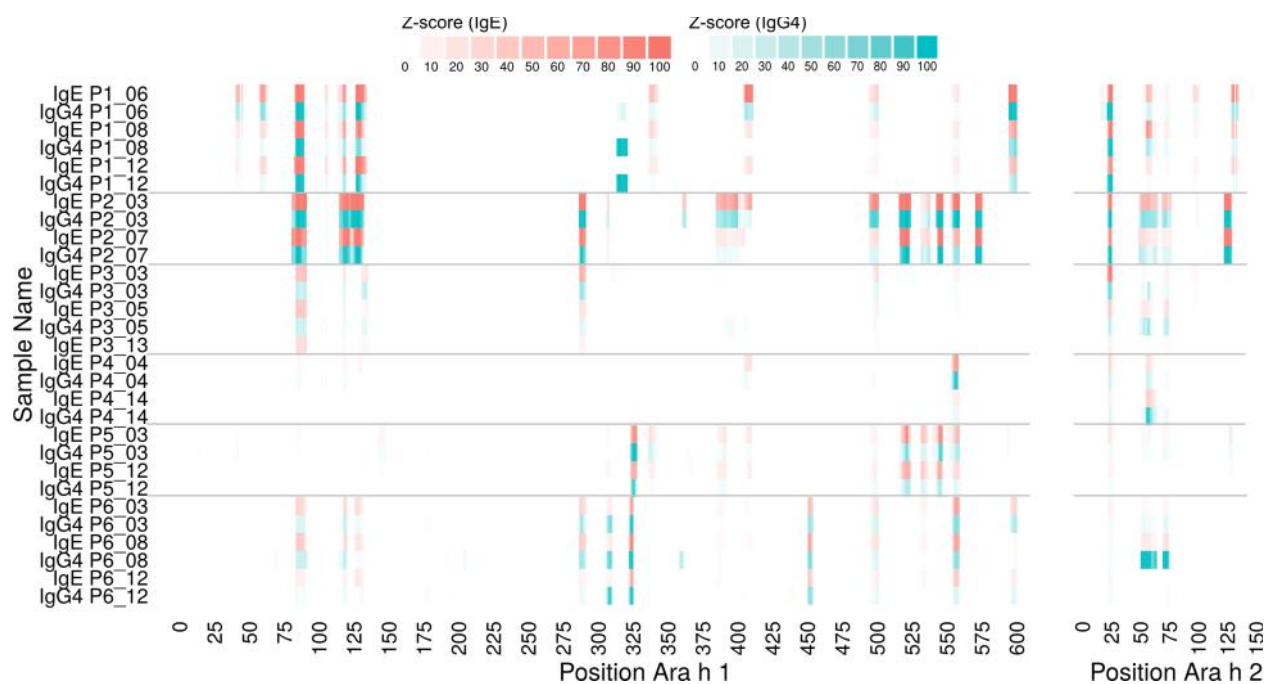


Figure E2: Reactivity of patient IgG4 and IgE respectively to peptides covering Ara h 1 and Ara h 2. The Z-score is reported at the starting position of a given peptide in the allergen sequence (x-axis) for both IgE (red) and IgG4 (blue). Each row indicates IgE or IgG4 channels from patient samples P1 through P6 at a certain year of sampling (e.g. P1_06 is Patient 1 sampled in 2006).

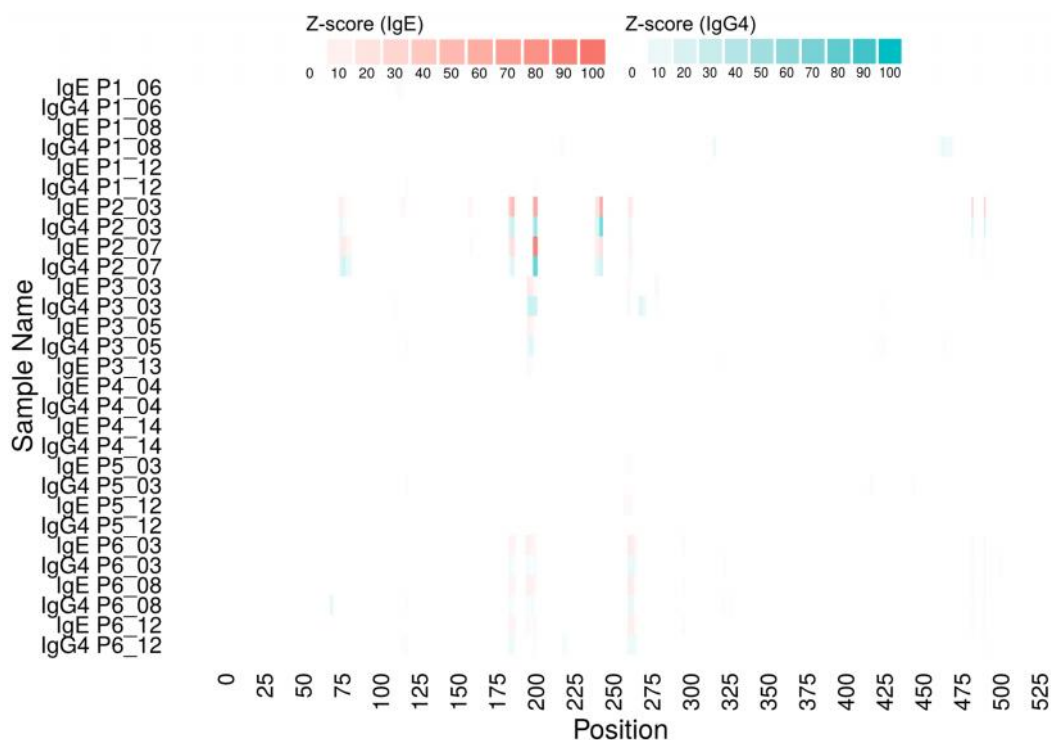


Figure E3. Reactivity to Ara h 3 derived peptides. The Z-score is reported at the starting position of a given peptide in the allergen sequence (x-axis) for both IgE (red) and IgG4 (blue). Each row indicates IgE or IgG4 channels from patient samples P1 through P6 at a certain year of sampling (e.g. P1_06 is Patient 1 sampled in 2006).

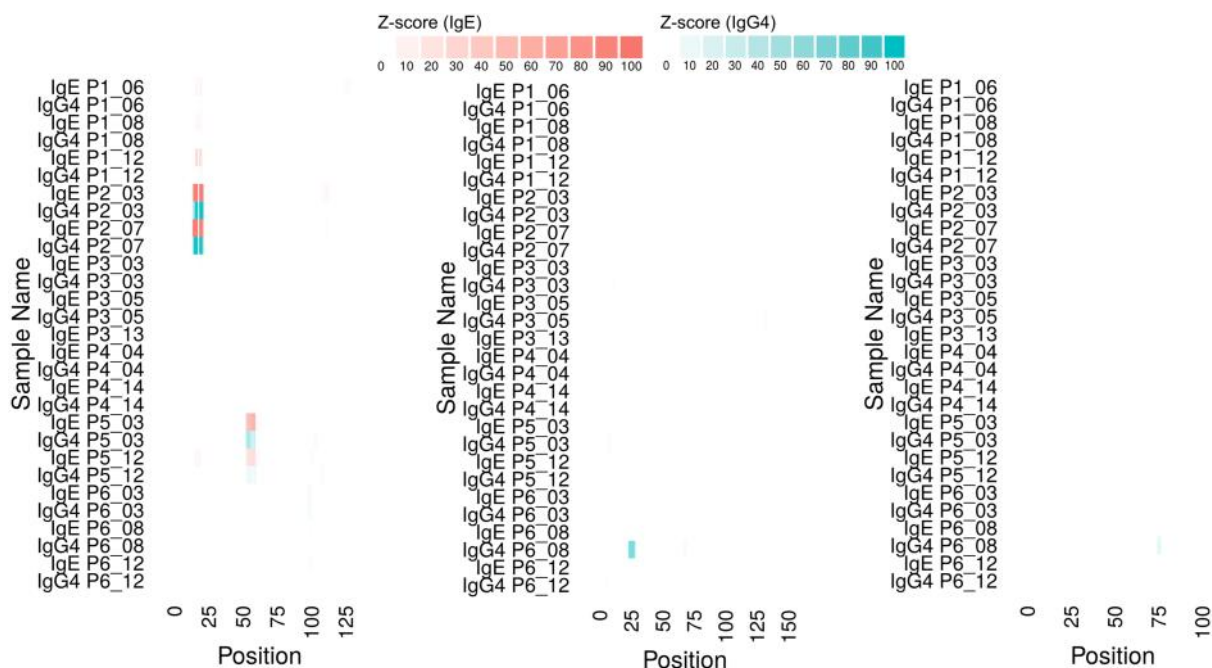


Figure E4. Similar to Figure E3, for Ara h 6 (left), Ara h 8 (middle) and Ara h 9 (right).

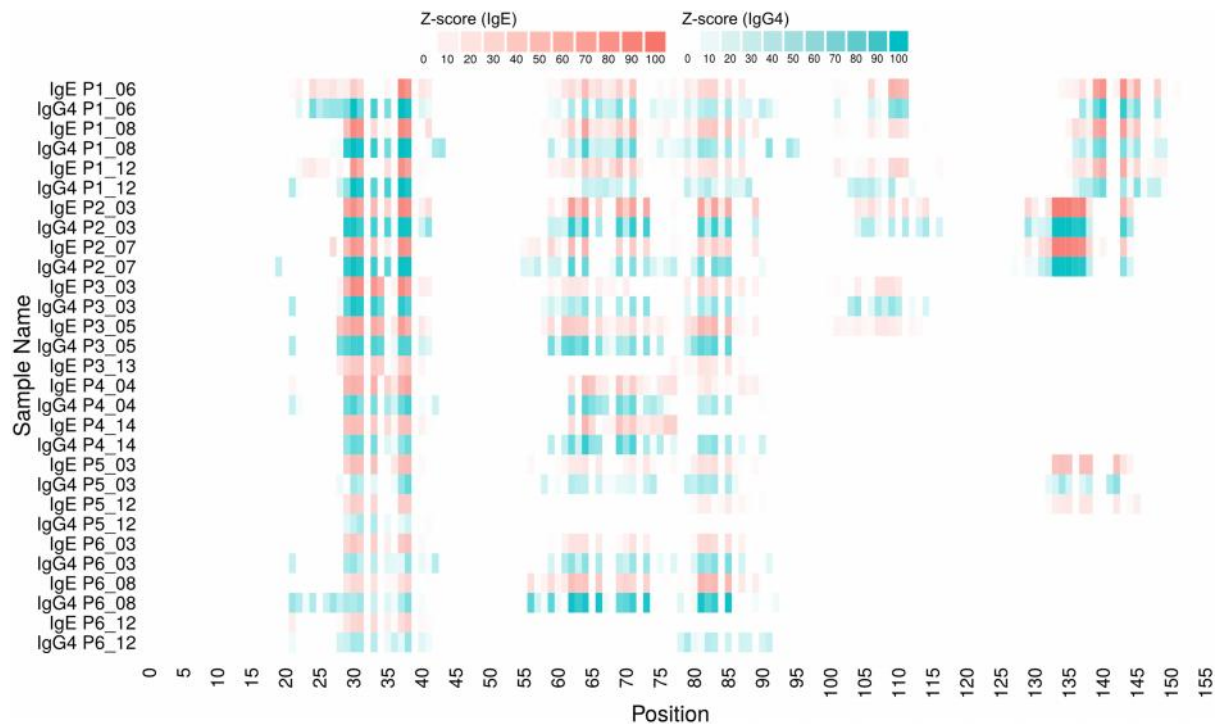


Figure E5. Alanine substitution effect in Ara h 2. The relative effect of substituting a given position to an alanine is reported as 0% to 100% loss of signal compared to the signal of the native 12-mer peptides. The substitution effects are reported as the median across multiple substitutions of the protein residue (x-axis) being expressed in overlapping binding peptides (see the Methods section for details). Each row indicates IgE or IgG4 channels from patient samples P1 through P6 at a certain year of sampling (e.g. P1_06 is Patient 1 sampled in 2006).

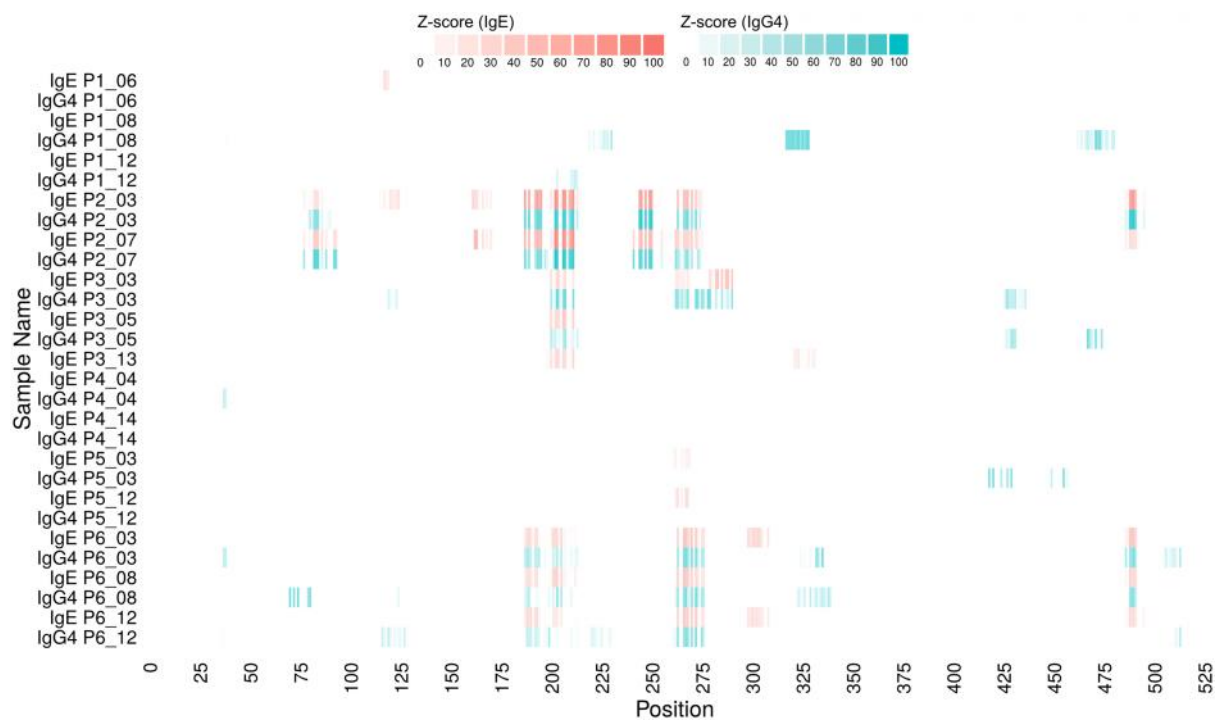


Figure E6. Similar to Figure E5, for Ara h 3.

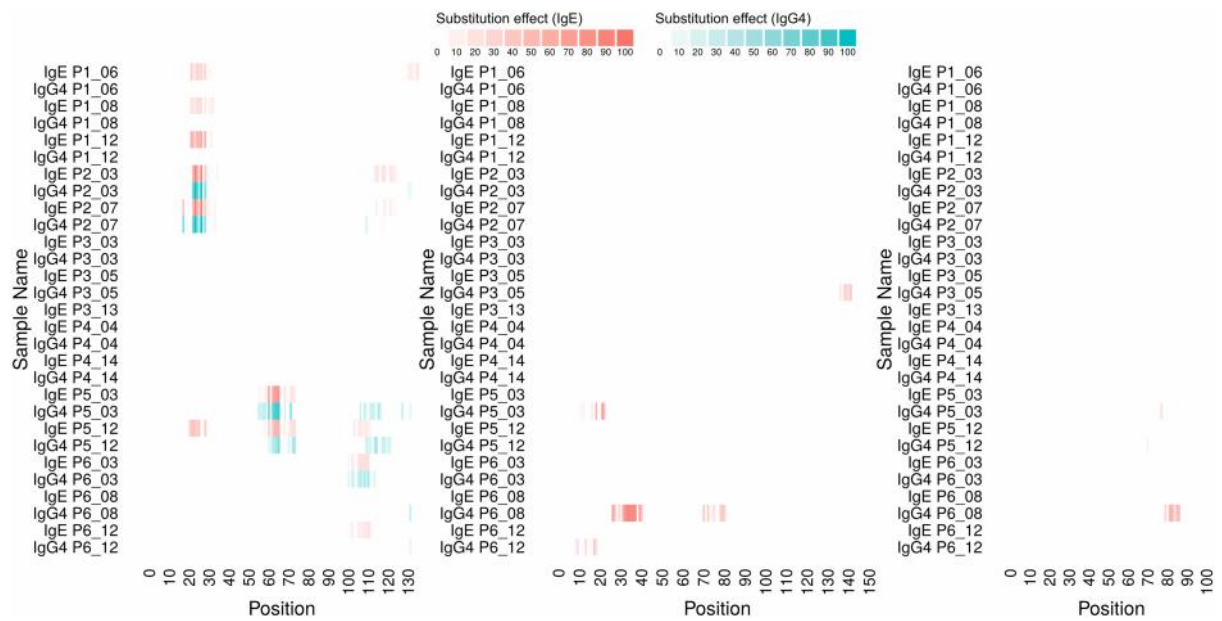


Figure E7. Similar to Figure E5, for Ara h 6 (left), Ara h 8 (middle) and Ara h 9 (right).

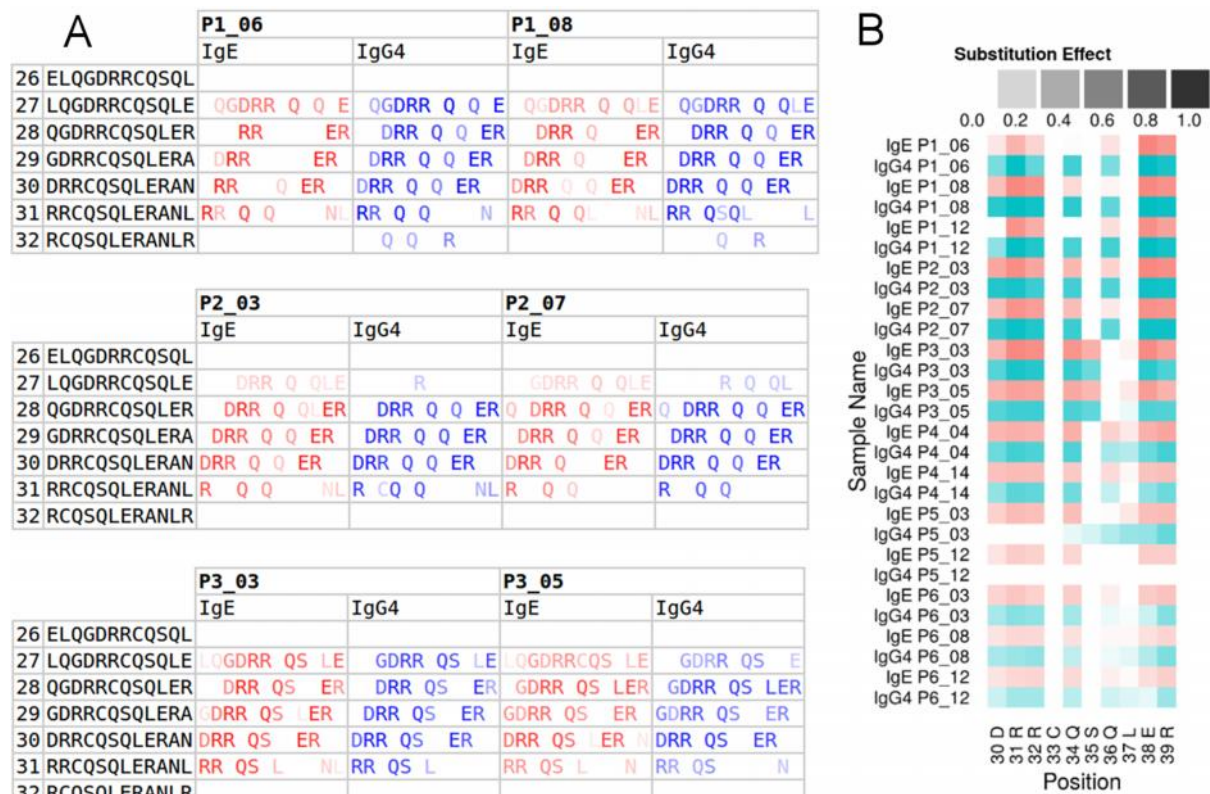


Figure E8: Alanine substitution effect for a sample epitope in Ara h 2 shown at high resolution. IgE effect is shown in red and IgG4 effect in blue. **(A)** Alanine substitution analysis of 7 individual peptides spanning the 26-44 region of Ara h 2. Rows indicate each sliding window of overlapping 12-mer peptides undergoing substitution. Columns indicate patients P1, P2 and P3 as well as the year of sampling, e.g. P1_06 indicate Patient P6 sampled in 2006. Peptide residues are shown in a zero to full intensity scale representing a 0-100% decrease in signal upon alanine substitution, see methods section for details. Blank cells depict peptides of no reactivity toward the sample. **(B)** Median substitution effects of Ara h 2 protein residues 30-39 represented in multiple overlapping 12-mer peptides.

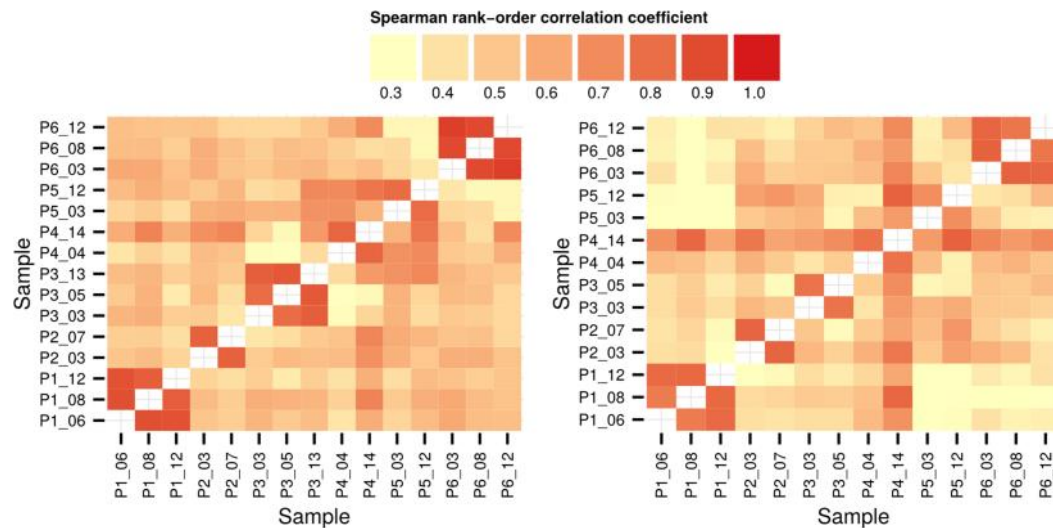


Figure E9. Correlation heatmaps of Spearman's rank correlation between substitution effects of allergen amino acids between samples. Shows result for IgE (left) and IgG4 (right). Only substitutions of immunoreactive peanut peptides in both samples are included.

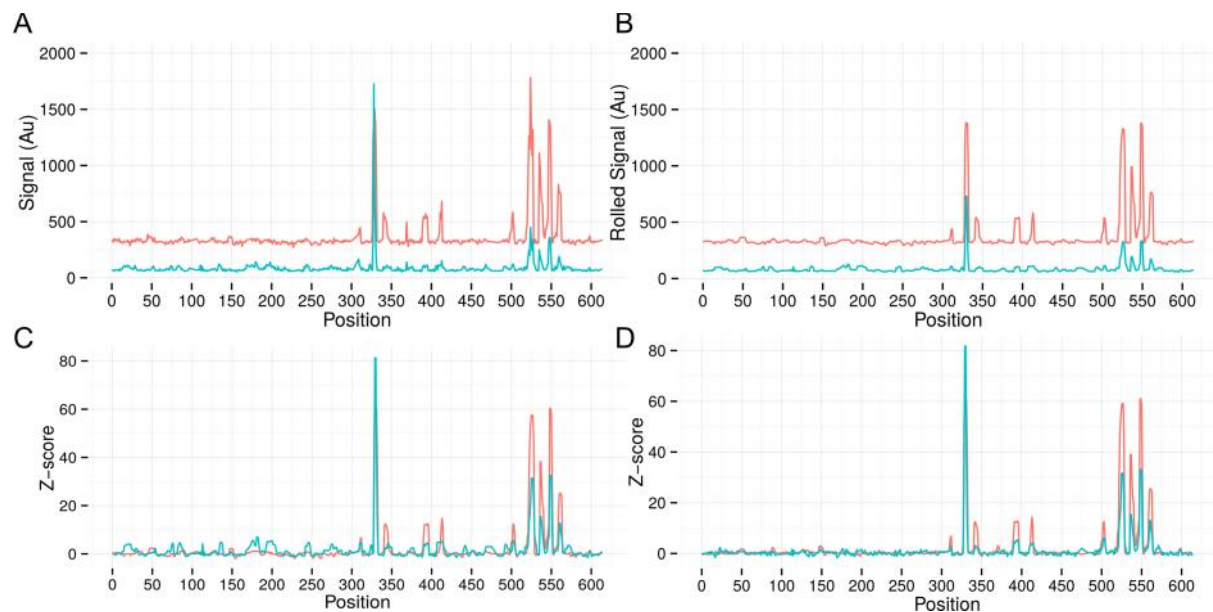


Figure E10. Illustration of signal normalization of IgE (red) and IgG4 (blue) signals of Ara h 1 peptides probed with sample P5_12. **(A):** Raw fluorescence signal (median of triplicates). **(B):** Signal smoothing using a rolling median across x-axis with a window size of 3. **(C):** Z-score of rolling median against baseline statistics calculated from non-relevant peptides. **(D):** Corrected Z-score: The Z-score of each peptide is subtracted by the maximum Z-score of identical peptides probed with control samples.

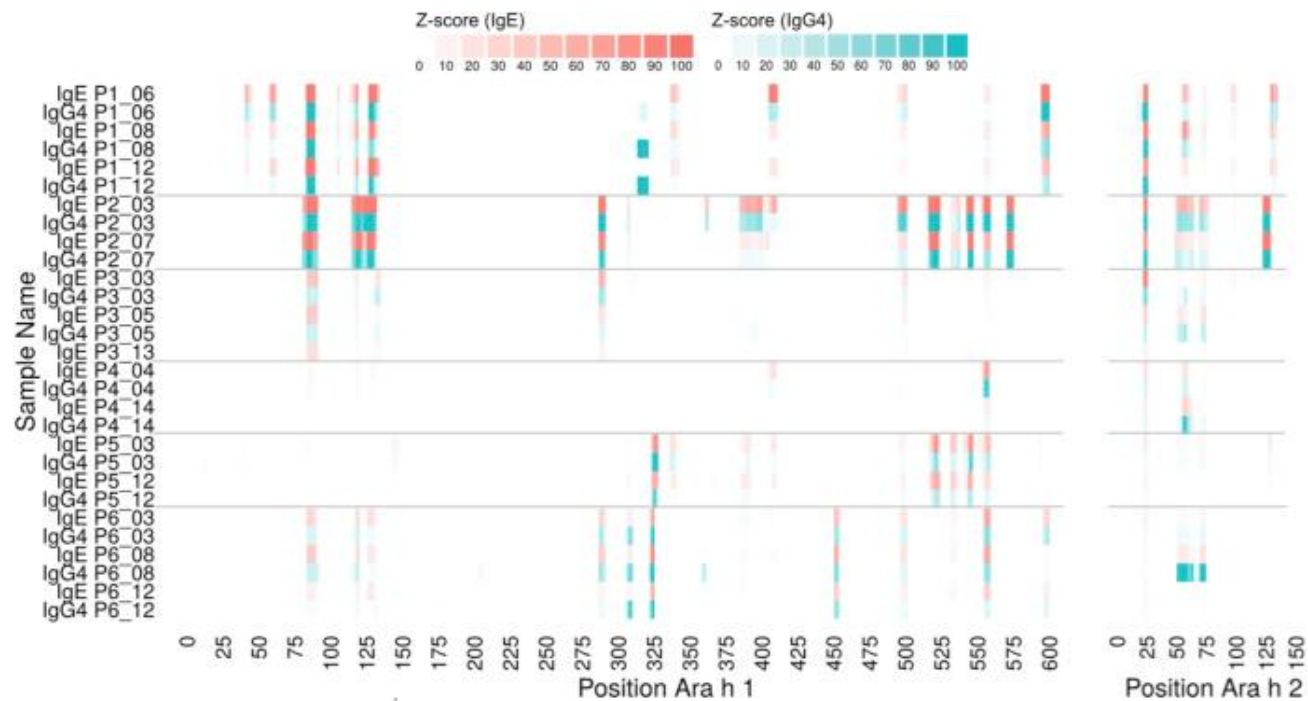


Fig E2.

Christian S. Hansen, Martin Dufva, Katrine L. Bøgh, Eric Sullivan, Jigar Patel, Thomas Eiwegger, Zsolt Szépfalusi, Morten Nielsen, Anders Christiansen

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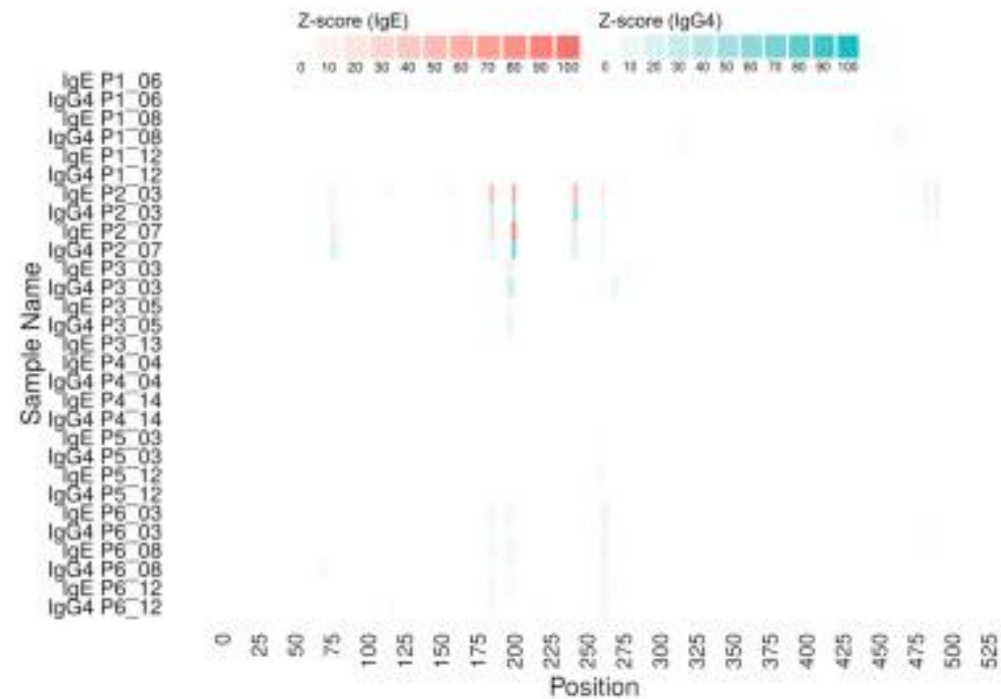


Fig E3.

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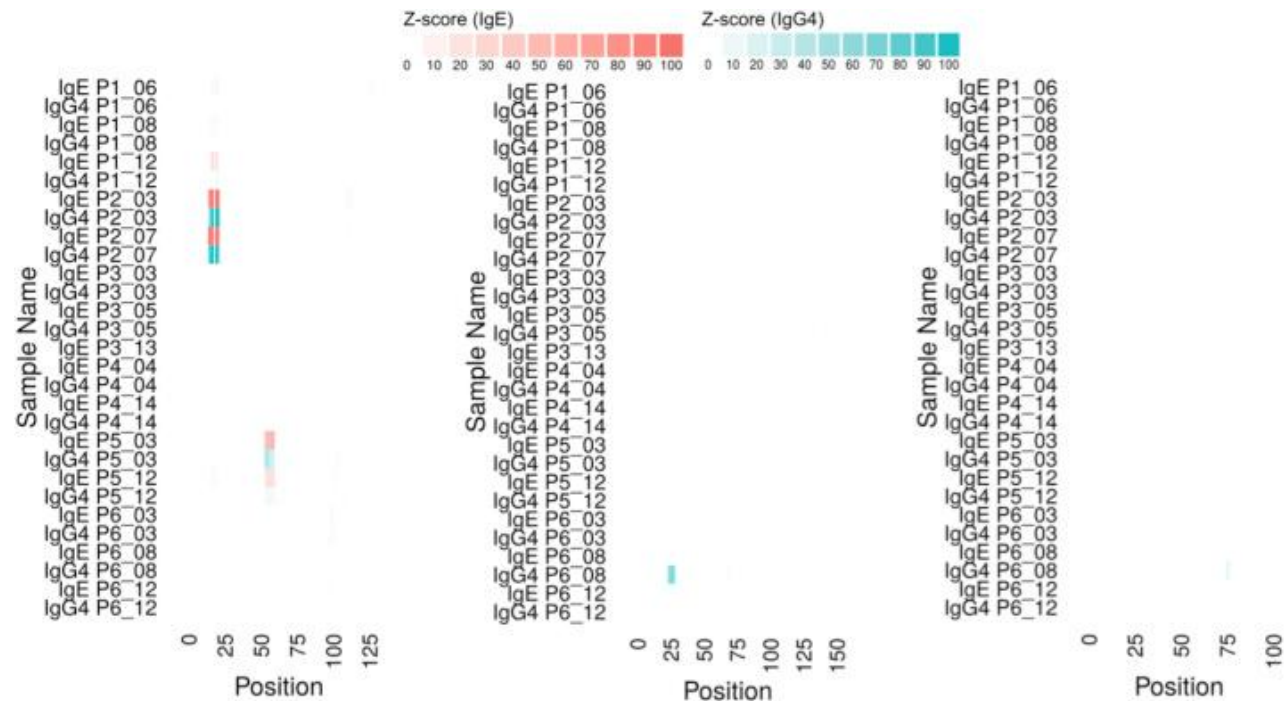


Fig E4.

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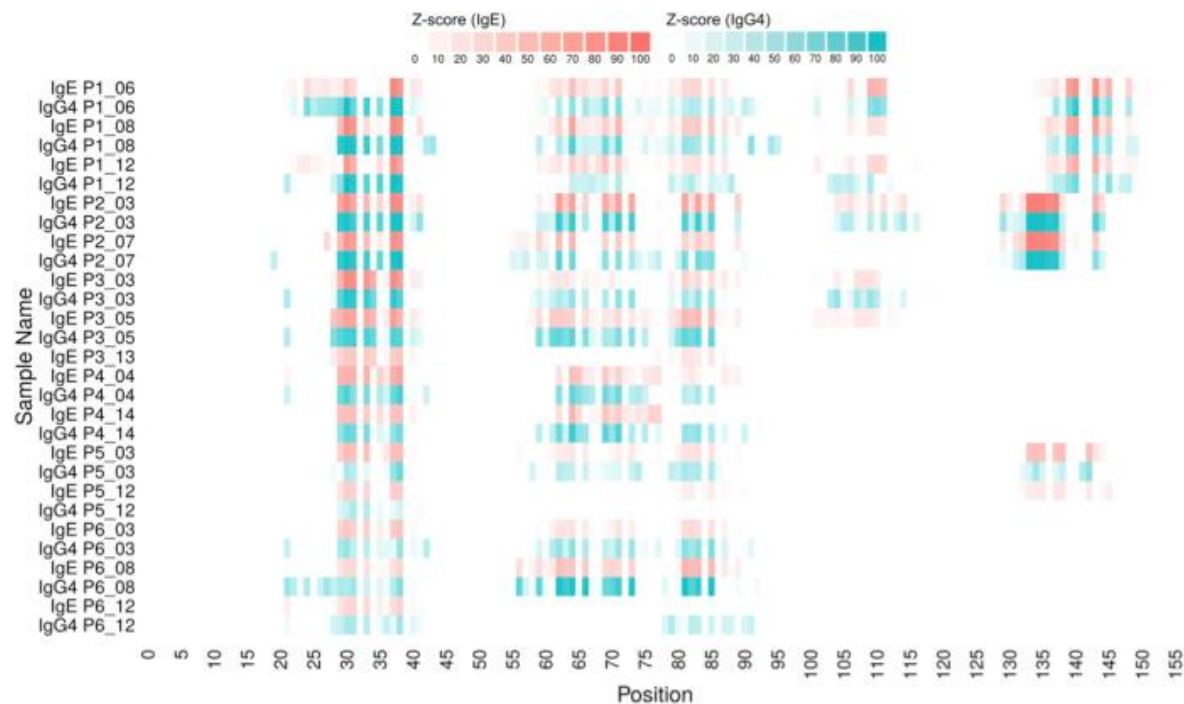


Fig E5.

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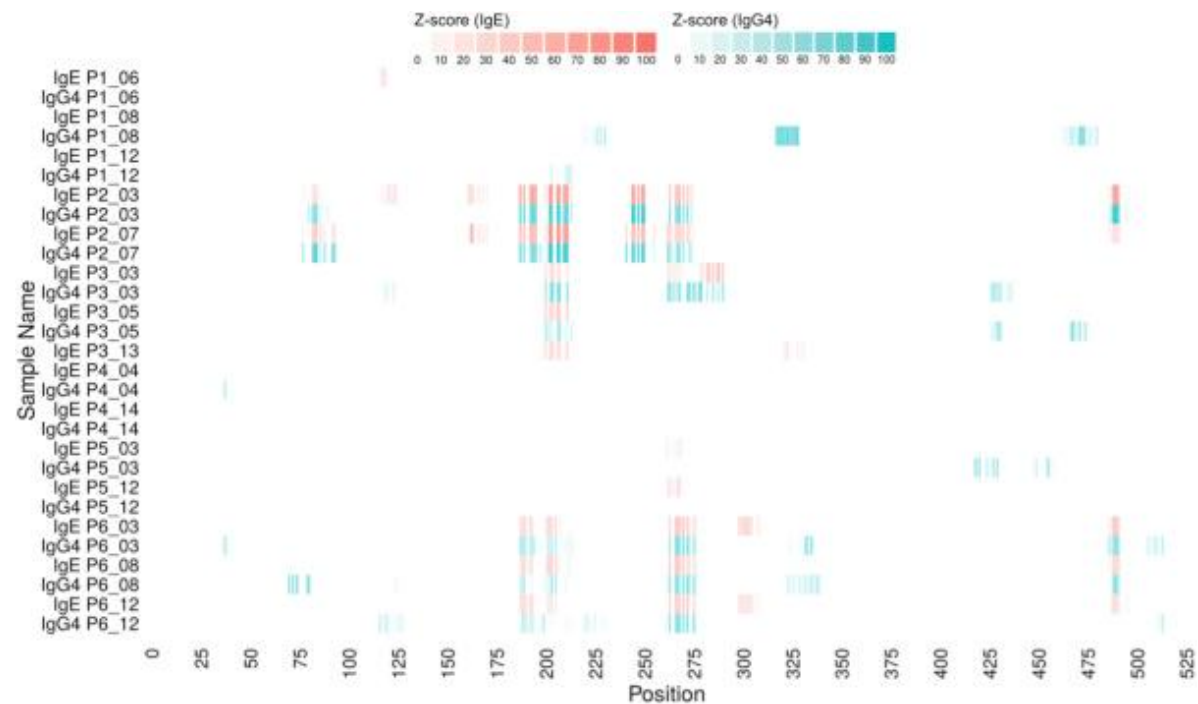


Fig E6.

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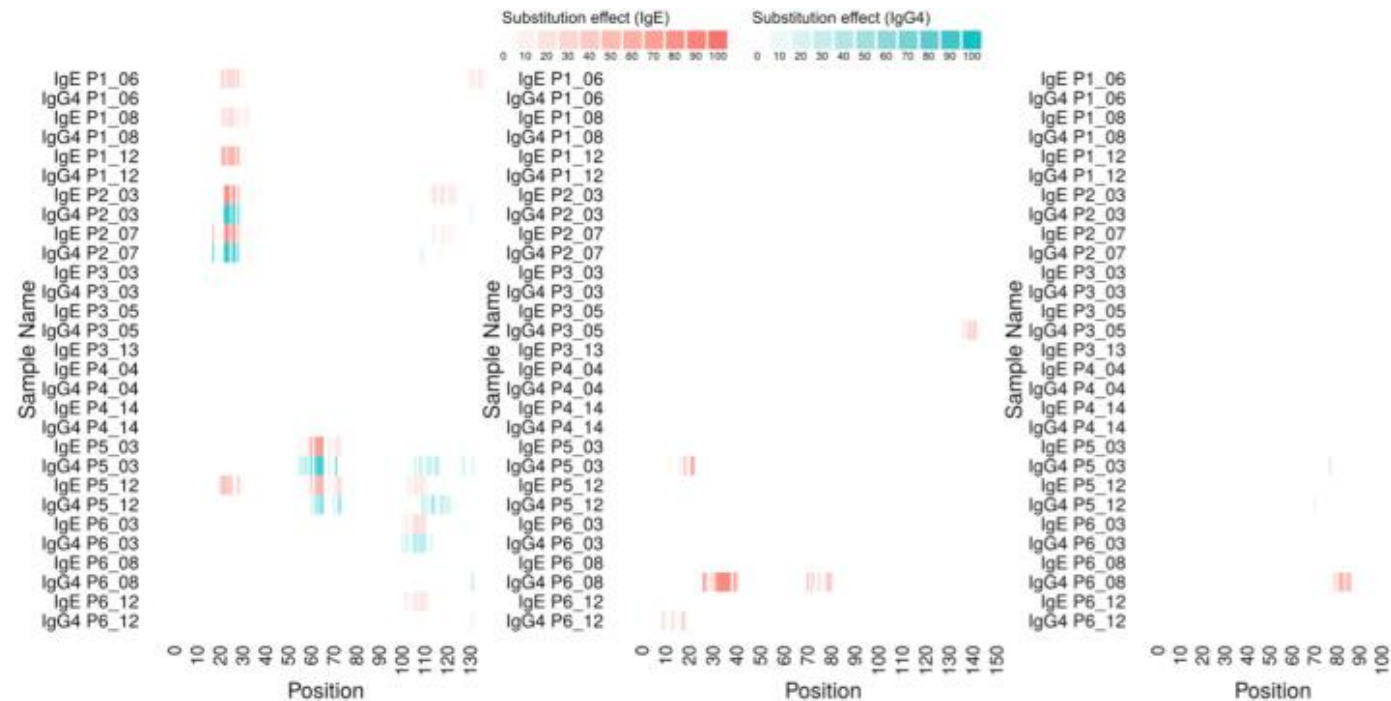


Fig E7.

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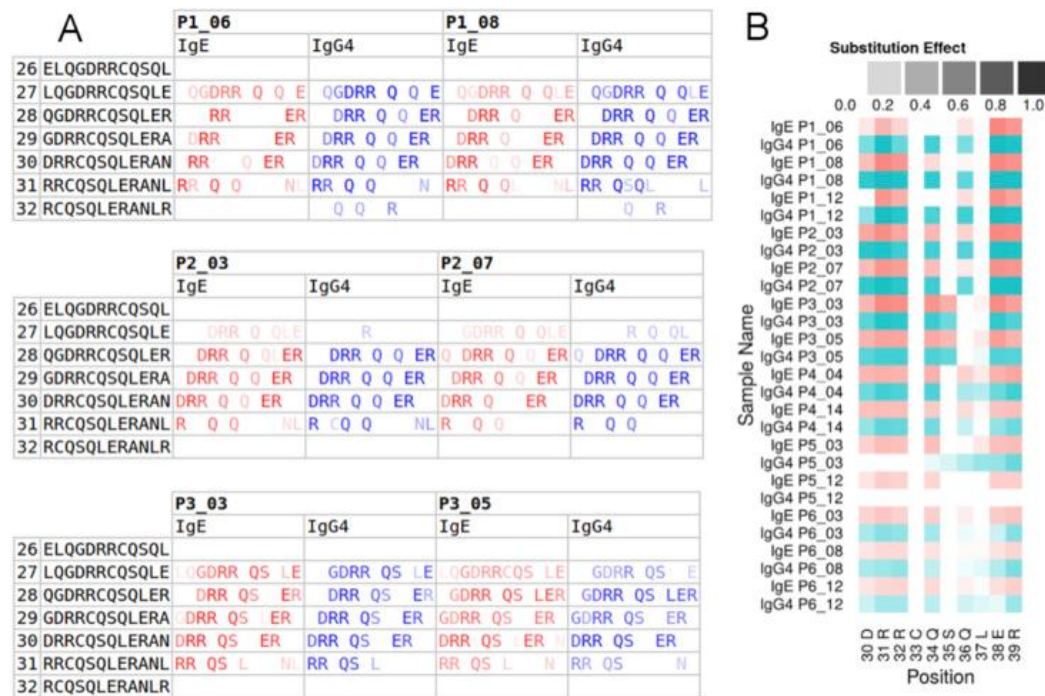


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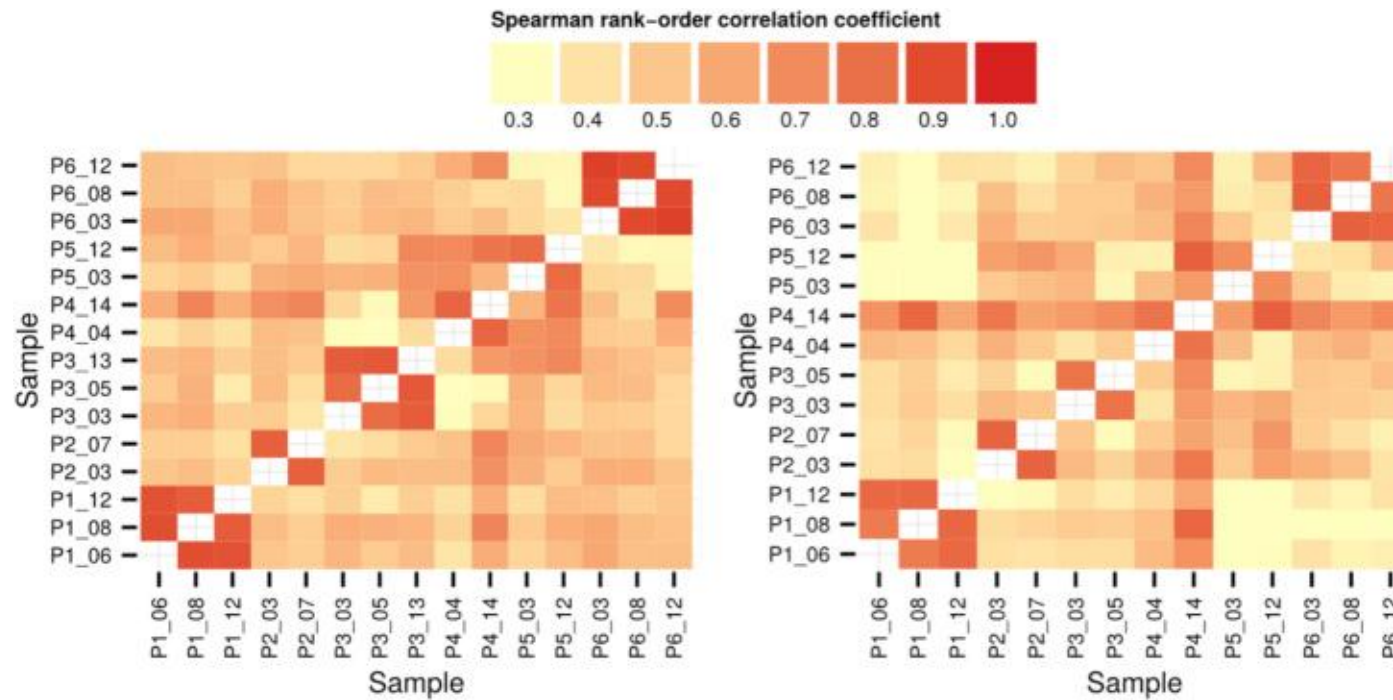


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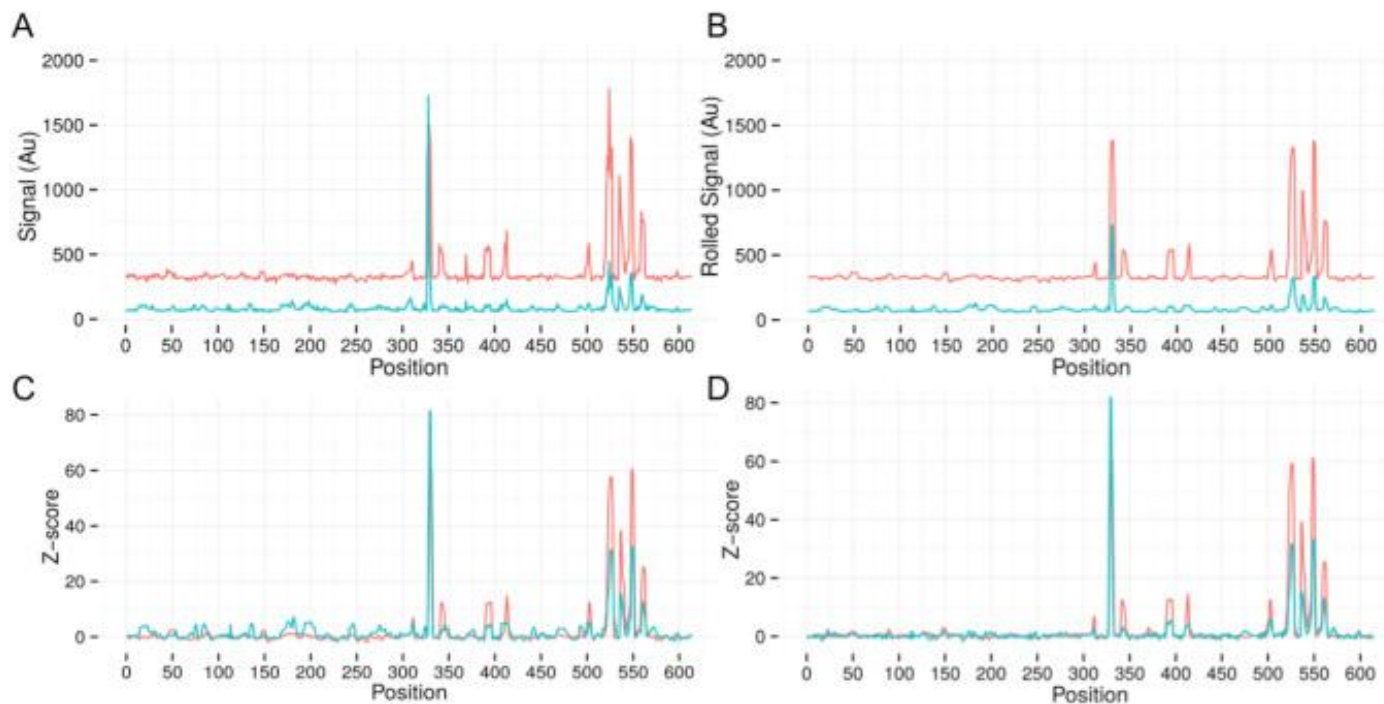


Fig E10.

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