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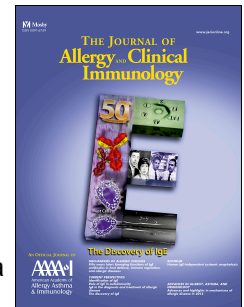
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TITLE: Isotype-Specific Agglutination-PCR (ISAP): a sensitive and multiplex method for measuring allergen-specific IgE

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CAPSULE SUMMARY: Isotype-Specific Agglutination-PCR (ISAP) is an operationally-simple multiplex assay for detecting specific IgE against allergen components in 1 μ L samples. We demonstrate that ISAP is more sensitive than ELISA and can yield results similar to ImmunoCAP.

LIST OF KEYWORDS: Component-resolved diagnostics (CRD); specific IgE (sIgE); qPCR; multiplex; sensitivity; low sample consumption; peanut allergy.

ABBREVIATIONS USED: Component-resolved diagnostics (CRD), Specific IgE (sIgE), Total IgE (tIgE), Immunoglobulin (Ig), Isotype-specific agglutination-PCR (ISAP), Proximity ligation assay (PLA), Antibody detection by agglutination-PCR (ADAP), Comprehensive ligation-based immunoglobulin quantification (CLIQ), POISED (Peanut oral immunotherapy study: safety, efficacy and discovery), ELISA (Enzyme-linked immunosorbent assay), Quantitative PCR (qPCR).

To the Editor:

Component-resolved diagnostics (CRD) are a rapidly-growing class of in-vitro tests for evaluating individuals thought to have allergic diseases. In contrast to using whole-allergen extracts, CRD employs individual, molecularly pure allergen components to measure allergen-specific IgE (sIgE), thereby helping to assess the risk of allergy to such allergens with improved accuracy¹. For example, the presence of sIgE against certain peanut components (e.g., Ara h 1, Ara h 2 and Ara h 3) indicates a higher risk for anaphylaxis than does anti-Ara h 8 sIgE (which can reflect cross-reactivity with birch pollen)¹. While ELISA-based CRD tests may lack analytical sensitivity, the alternative more sensitive assays currently employed clinically (e.g., ImmunoCAP) are not multiplexible and may omit certain critical allergens². These problems can be mitigated by collecting more blood for additional tests. However, this approach can be problematic when studying small animals or testing young children.

Here we report Isotype-Specific Agglutination-PCR (ISAP), a highly sensitive and multiplexible approach for measuring allergen-specific immunoglobulins (Igs) in 1 μ L of sample (Fig 1, A). ISAP employs chemically-synthesized allergen-DNA and secondary antibody-DNA conjugates (see Fig E1 in this article's Online Repository at www.jacionline.org). Each DNA conjugate bears either the 5' or 3' portion of a split DNA barcode (Fig 1, A). Upon binding to the target Ig in the sample, the allergen-DNA and secondary antibody-DNA conjugates are agglutinated into close proximity. The addition of a short complementary bridge oligo and DNA ligase reunites the two halves of the barcode to create a full-length DNA amplicon, which can then be quantified by qPCR. The amount of the amplicon directly reflects the quantity of analyte within the sample. Importantly, in the absence of the specified allergen-specific Ig, the two DNA conjugates will neither ligate nor amplify by PCR. By requiring the presence of the analyte to generate signal, this "turn-on" mechanism circumvents the washing or DNA purification steps needed to remove unbound secondary reporters in other formats such as immuno-PCR^{3,4}. In principle, these features allow ISAP to represent a CRD assay with enhanced sensitivity, multiplex capability, and an operationally simple workflow.

To establish proof-of-concept for the detection of allergen-specific IgE (sIgE), we prepared a dilution series of: 1) anti-OVA sIgE, 2) anti-OVA sIgG and 3) total IgE (control). The three samples were then analyzed with

an ISAP assay designed to detect anti-OVA sIgE. As expected, concentration-dependent signal arose only for the sample containing anti-OVA sIgE (Fig 2, A). Next, we performed a head-to-head comparison of ISAP with a standard ELISA, which showed a markedly (~800-fold) increased analytical sensitivity of ISAP compared to ELISA (Fig 2, B).

In addition to sIgE, total IgE and total anti-allergen sIg levels (of all Ig classes) are also of interest in characterizing allergic responses. To create an assay capable of measuring these parameters, we integrated ISAP with two other PCR-based methods: the Proximity Ligation Assay (PLA)⁵ and Antibody Detection by Agglutination-PCR (ADAP)⁶ (Fig 1, B and C). PLA employs two secondary antibody-DNA conjugates to detect total IgE (tIgE) whereas ADAP uses two allergen-DNA conjugates to detect all allergen-specific Igs (e.g., IgE, IgG, IgM). The integrated assay, termed CLIQ (Comprehensive Ligation-based Immunoglobulin Quantification), converts sIgE, tIgE and all sIgs of any isotypes into distinct DNA amplicons that can be independently interrogated with unique primer pairs (Fig 1, D).

To validate this concept, we re-analyzed the anti-OVA sIgE, anti-OVA sIgG and total IgE (control) dilution series with CLIQ (see Fig E2 in this article's Online Repository at www.jacionline.org). For instance, Fig E2, A shows that both the PLA (which measures total IgE, solid squares) and the ISAP (which measures sIgE, open triangles) portions of CLIQ lacked signals when used to assess anti-OVA sIgG; only the ADAP portion of CLIQ (which measures total anti-OVA sIgs, solid circles) shows concentration-dependent signals. The data in Fig E2 indicate that each of the three PCR-based methods selectively detected its specified target in an integrated assay without cross-reactivity.

Next, we sensitized mice with OVA⁷ and analyzed their antibody responses using CLIQ. CLIQ demonstrated elevations in serum and blood levels of tIgE and total anti-OVA sIg at d7 and sIgE at d14 in OVA-sensitized mice, but not in PBS-treated control mice (see Fig E3, A and C, and Fig E4 in this article's Online Repository at www.jacionline.org). ELISA also detected an elevation in serum sIgE at d14 in the same OVA-sensitized mice (see Fig E3, B in this article's Online Repository at www.jacionline.org). These results demonstrate substantial equivalence between the CLIQ and ELISA methods in this setting, in which strong sIgE responses are induced.

To probe the sensitivity of CLIQ in settings with low levels of serum sIgE, we sensitized mice epicutaneously with peanut oil. The sensitized mice were later challenged with peanut extract to attempt to induce anaphylaxis. Despite the strong systemic reaction to peanut challenge (see Fig E5 in this article's Online Repository at www.jacionline.org), peanut sIgE was undetectable by ELISA (Fig 2, D and Fig E6). However, when we assayed serum from these mice with CLIQ (using multiple peanut components as antigens: Ara h 1, Ara h 2 and Ara h 3), CLIQ, but not ELISA, detected increases in tIgE and Ara h 1 sIgE after peanut oil sensitization in BALB/c or C57BL/6 mice (Fig 2, C and see Figs E7 and E8 in this article's Online Repository at www.jacionline.org). By contrast, CLIQ did not detect such changes in antibody-deficient *Jh*^{-/-} and *Rag*^{-/-} mice after we attempted to induce peanut sensitization⁸ (Fig 2, C). These results show that the enhanced analytical sensitivity of CLIQ can detect potentially disease-relevant levels of sIgE which may be undetectable by traditional means like ELISA.

Finally, we employed CLIQ to analyze plasma obtained from 20 peanut-allergic subjects upon their enrollment into the IRB-approved POISED trial (ClinicalTrials.gov Identifier: NCT02103270)⁹. CLIQ results displayed substantial positive correlations with ImmunoCAP data from the same specimens (Fig 2, E and see Figs E9, E10 and E11 in this article's Online Repository at www.jacionline.org).

It is important to emphasize that no assay for sIgE can be used, in isolation, to establish the diagnosis of a clinically relevant allergy. However, such testing, if used appropriately, can identify those who have been sensitized to specific allergens or their components and therefore can confirm that these individuals are potentially at risk for exhibiting sIgE-associated clinical allergies. Although it is not possible to fully prevent the misuse of any diagnostic assay, and this is a particular concern with highly sensitive assays, our data suggest that, as with the ImmunoCAP assay, appropriate selection of the assay cutoff value will permit the CLIQ assay to be used to identify potentially clinically relevant levels of sIgE against allergen components.

In summary, ISAP is a sensitive and specific method for multiplex detection of isotype-specific sIgE against specific allergen components in very small (1 μ L) sample volumes. Integration of ISAP with PLA and ADAP to create the CLIQ assay greatly expands the information which can be obtained in a single assay. We envision

that the much lower sample consumption and improved sensitivity of this assay will prove useful for allergy research and diagnostics, and for the management of allergy patients.

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MATERIALS AND METHODS:

Materials and Methods are described in the "Supplemental Material" section.

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FIGURE LEGENDS

Figure 1. Principle scheme of PCR-based antibody quantification methods. (A) ISAP for quantification of sIgE. Samples containing sIgE are first incubated with allergen-DNA and antibody-DNA (in this case, anti-IgE antibody-DNA) conjugates. Upon formation of immune complexes consisting of sIgE, allergen-DNA and anti-IgE-DNA, the DNA components of the conjugates are brought into close proximity, and the addition of a short bridge oligo and DNA ligase joins the two halves of DNA into a full length amplicon. Real-time qPCR is then used to quantify the abundance of the ligated DNA amplicons, which reflects the level of sIgE in the sample. (B) PLA for quantification of total IgE (tIgE). Samples are incubated with two anti-IgE-DNA conjugates that each bear half of a full-length DNA amplicon. (C) ADAP for quantification of all anti-allergen immunoglobulins (IgE, IgG, IgM, etc.). This procedure is performed as before, but with two allergen-DNA conjugates. (D) Integration of ISAP, PLA and ADAP into a single assay, termed CLIQ. The sample is incubated with two allergen-DNA and two anti-IgE-DNA conjugates followed by ligation. The reconstituted DNA amplicons can be interrogated independently by dedicated primer pairs in the different wells of a 96- or 384-well plate.

Figure 2. Multiplex and sensitive detection of allergy-related immunoglobulins by PCR. (A) Serially-diluted anti-OVA sIgE, anti-OVA sIgG and total IgE (control) analyzed with ISAP (Δ Ct [Delta Cycle

threshold], a standard way to report qPCR signal=Ct value of buffer-only control minus that of sample). **(B)** Aliquots of the same anti-OVA sIgE dilution series assayed by ISAP (Δ Ct) and ELISA (AU=arbitrary units). **(C)** CLIQ profiles of immunoglobulin responses to peanut sensitization in BALB/c wild type mice, BALB/c-*Rag*^{-/-} (Rag KO) mice and BALB/c-*Jh*^{-/-} (Jh KO) mice (n=5/group) sensitized to peanut epicutaneously. CLIQ results are expressed as the relative fold change (value after sensitization divided by value before sensitization) (* $P < .05$ for values before vs. after sensitization). The h1, h2 and h3 labels in the x-axis refer to Ara h1, Ara h2 and Ara h3, respectively. **(D)** ELISA analysis of sera from the same 5 BALB/c mice analyzed by ISAP in **C**. No statistically significant differences in levels of anti-peanut sIgE were observed by ELISA before vs. after sensitization. **(E)** Correlation of ISAP and ImmunoCAP results ($R=0.83-0.96$, $P < .05$). 1 μ L each of 20 baseline (pre oral immunotherapy) plasma specimens from participants in the POISED trial analyzed by ISAP (x-axis) vs. ImmunoCAP (y-axis).

DNA sequence design

Sequence name	Sequence	Note
Seq1A	CAGGTAGTAGTACGTCTGTTTCACGATGAGACTGGATGAA	5'-thiol
Seq1B	TCACGGTAGCATAAGGTGCAAGATAATACTCTCGCAGCAC	5'-phos 3'-thiol
Seq2A	GGCCTCCTCCAATTAAAGAATCACGATGAGACTGGATGAA	5'-thiol
Seq2B	TCACGGTAGCATAAGGTGCAGTACCCAAATAACGGTTCAC	5'-phos 3'-thiol
Seq3A	GGATCACTCCAACCTAGACTATCACGATGAGACTGGATGAA	5'-thiol
Seq3B	TCACGGTAGCATAAGGTGCAGTTATATCTGCCACTGTCAC	5'-phos 3'-thiol
Seq4A	AGAGTCCACTTCCCATAATGTCACGATGAGACTGGATGAA	5'-thiol
Seq4B	TCACGGTAGCATAAGGTGCACGGTACTGTCAGCATAGTTC	5'-phos 3'-thiol
Seq1F	CAGGTAGTAGTACGTCTGTT	
Seq1R	GTGCTGCGAGAGTATTATCT	
Seq2F	GGCCTCCTCCAATTAAAGAA	
Seq2R	GTGAACCGTTATTTGGGTAC	
Seq3F	GGATCACTCCAACCTAGACTA	
Seq3R	GTGACAGTGGCAGATATAAC	
Seq4F	AGAGTCCACTTCCCATAATG	
Seq4R	GAAGTATGCTGACAGTACCG	
Connector	CTACCGTGATTCATCCAG	

FIGURE 1

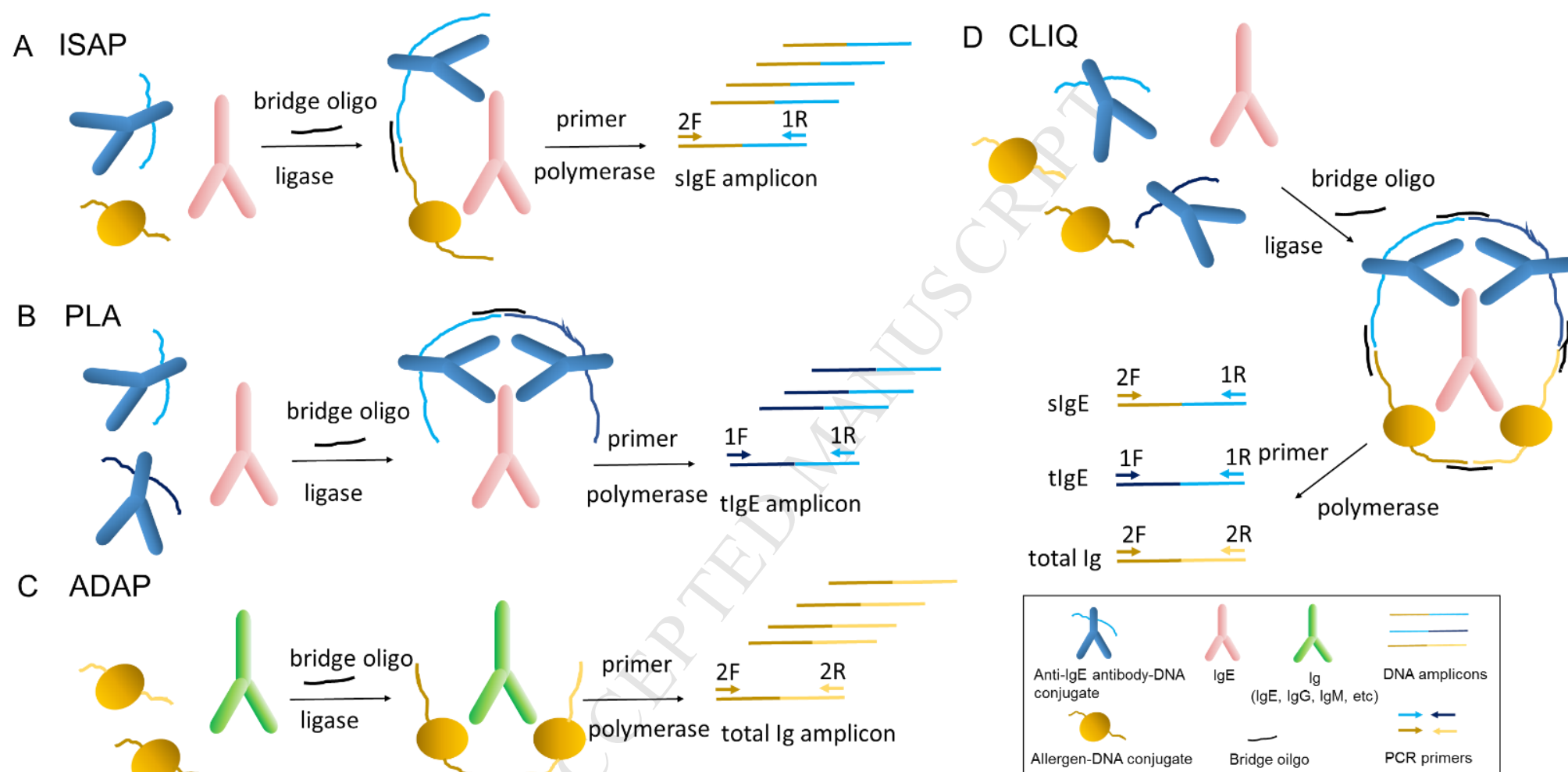
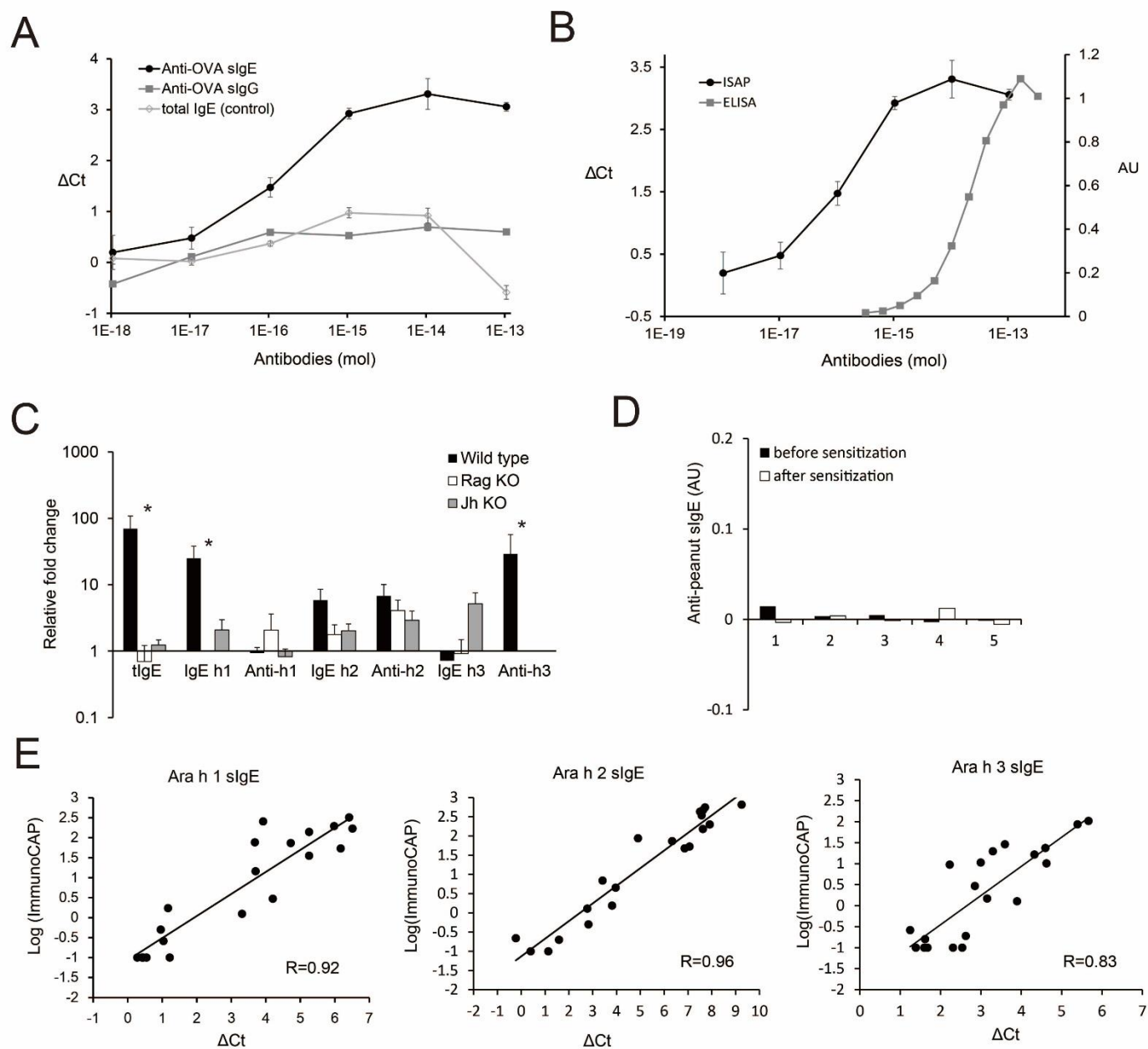


FIGURE 2



ONLINE REPOSITORY

METHODS

Materials and Reagents. Chemicals (including KCl, MgCl₂, Tris, EDTA, NAD, Triton X-100 and bovine serum albumin) were purchased from Sigma Aldrich (St. Louis, MO) or DMSO, DTT, sulfo-SMCC and dNTP from Life technologies (Carlsbad, CA). All oligonucleotides were manufactured by Integrated DNA Technologies (abbreviated as IDT, Redwood City, CA). The specific sequences for all oligonucleotides are provided in the DNA sequence table immediately below. Anti-ovalbumin IgE was from AbD Serotec (#MCA2259, Hercules, CA), IgE isotype control (also referred to as total IgE control [tIgE]) was from Biolegend (#401701, San Diego, CA) and anti-ovalbumin IgG was from Abcam (ab17293, Cambridge, United Kingdom). OVA protein was from Life Technologies (#77120) and Ara h1 (#LTN-AH1-1), Ara h2 (#RP-AH2-1) and Ara h3 (#NA-AH3-1) were from Indoor Biotechnologies (Charlottesville, VA). Anti-mouse IgE antibody was from Abcam (#ab19967), anti-human IgE antibody was from AbD Serotec (#STAR147), DNA ligase was from EpiCentre (#A8101, Madison, WI) and DNA polymerase (#10966018) and SYBR qPCR 2X master mix (#4385610) were from Thermo Fisher (Waltham, MA). Other reagents and assay kits are described in other parts of the Methods section.

DNA sequences with an A in their name have a 5' thiol-group (5'-thiol modifier C6 S-S) to enable conjugation to proteins, whereas DNA sequences with a B in their name have 5' phosphorylation to enable ligation and a 3' thiol-group (3'-thiol modifier C3 S-S) to enable conjugation. DNA sequences and primers were optimized to minimize formation of secondary structures and primer dimers while maximizing amplification efficiency.

Analysis of human plasma. Plasma samples from patients with peanut allergy were obtained as part of their enrollment into an institutional review board-approved clinical trial of oral immunotherapy (OIT) in children and adults with peanut allergy (POISED; ClinicalTrials.gov Identifier: NCT02103270). Peanut allergy was defined as having a reaction to a double-blind, placebo-controlled food challenge to peanut (with reactions elicited with ≤ 500 mg of peanut protein) and a positive skin prick test response to peanut (wheal ≥ 5 mm).

OVA sensitization. Sensitization of mice was achieved by administration of OVA with alum. The protocol was performed as described in ¹, with minor modifications. Briefly, mice (n=5/group) were immunized intraperitoneally with 100 μ g OVA (Grade V; Sigma-Aldrich, St Louis, MO), 100 ng of *B pertussis* toxin (List Biologicals, Campbell, CA), and 50 μ L Imject Alum (Thermo Scientific, Waltham, MA) in PBS in a total volume of 100 μ L on days 0 and 14. Control mice (n=5) were inoculated similarly with 100 μ L of a solution containing 100 ng of *B pertussis* toxin and 50 μ L Imject Alum in PBS, but without OVA. Serum and whole blood samples were collected via retro-orbital bleeding on days 0, 7, 14, and 21. All serum samples were stored at -80°C until used. Whole blood samples were diluted 1:1 in 10 mM EDTA and 1X PBS right after collection to prevent clotting. Whole blood samples were stored at -80°C until used.

Sensitization with peanut oil and intraperitoneal challenge with peanut protein. The

abdominal skin of BALB/c wild type mice, C57BL/6 wild type mice, BALB/c-*Jh*^{-/-} mice and BALB/c-*RAG*^{-/-} mice (all n=5/group) was shaved and then depilated with Veet (Reckitt Benckiser, Slough, United Kingdom), followed by washing with PBS, and the mice then were sensitized once a week with 200 μ L of peanut oil (#570600, Golden Peanut Company, Dawson, Georgia) for 6 weeks. Per the manufacturer, the peanut oil is crude oil without any filtration or refinement, and Jablonski, et al. confirmed the presence of peanut protein (approx. 400 ppm) in this oil using colorimetric assays.² After application, each mouse was gently held by the investigator for 1 min for the peanut oil to be absorbed, and then 3-5 mice treated in this way were housed in the same cage. BALB/c and C57BL/6 mice were then challenged i.p. with 5 mg of peanut protein (extracted from defatted peanut flour as previously described (Byrd Mill, Ashland, Virginia)³ administered in 300 μ L of PBS, or received PBS alone as a control. Rectal measurements of body temperature were performed immediately before (time 0) and at different time points for up to 60 minutes after challenge, as described above. Serum samples (stored at -80°C until used) were collected on day 0 via retro-orbital bleeding and on day 42 via cardiac puncture after sacrificing the mice by inhalation of CO₂.

ELISA analysis. The ELISA to measure specific IgE (sIgE) was performed as described in ¹ with minor modifications. Briefly, OVA was deposited on ELISA plates to capture and detect anti-OVA IgE in samples of purified IgE or in serum from OVA-sensitized mice. The amount of surface-bound IgE was quantified by detecting absorbance after treatment with biotinylated secondary anti-mouse IgE and streptavidin horseradish peroxidase conjugate (SA-HRP). The SA-HRP reacts with its substrate TMB (3,3',5,5'-Tetramethylbenzidine, Thermo Fisher) to yield a colorimetric reaction that can be quantified by a plate reader. For the detection of peanut-specific IgE, whole peanut extract (#F171, Greer Laboratories, Lenoir, NC) was coated on ELISA plates and anti-peanut IgE was detected as described above. This ELISA method should detect all peanut specific IgE. To further measure Ara h 1 sIgE, we followed the protocol of Smit, et al., and Van Wijk, et al. as detailed in ^{4,5}. Briefly, 96 well plates (Costar, Washington, D.C.) were coated with rat anti-mouse IgE (#553413, BD Biosciences, San Jose, CA) at 4°C overnight. Following blocking with 5% bovine serum albumin (Sigma-Aldrich, St. Louis, MO) in PBS for 2 h at room temperature, serum (1:8 dilution) was added for 2 h at room temperature. Digoxin (DIG) conjugated recombinant Ara h1 (10 μ g/mL, #LTN-AH1-1, Indoor Biotechnologies, Charlottesville, VA) then was added and incubated for 2 h at room temperature. DIG was conjugated according to the manufacturer's instructions (#55865, digoxigenin NHS-ester, Sigma-Aldrich). After incubation for an additional 2 h at room temperature with alkaline phosphatase-conjugated anti-DIG antibody (Sigma-Aldrich), p-nitrophenylphosphate (Sigma-Aldrich) was added as a substrate and OD 405 nm was measured.

ImmunoCAP analysis. Was performed by Phadia, Thermo Fisher (Waltham, MA).

Synthesis of allergen-DNA and antibody-DNA conjugates. OVA was obtained from Life Technologies (#77120, Charlottesville, VA). Ara h1 (#LTN-AH1-1), Ara h2 (#RP-AH2-1) and Ara h3 (#NA-AH3-1) were purchased from Indoor Biotechnologies (Charlottesville, VA). Allergen (OVA, Ara-h1, Ara-h2 and Ara-h3)-DNA conjugates were synthesized by suspending purified or recombinant

protein in Reaction Buffer (1 mg/mL protein in 55 mM sodium phosphate, 150 mM sodium chloride, 20 mM EDTA, pH 7.2). SMCC (succinimidyl 4-(N-maleimidomethyl)cyclohexane-1-carboxylate, Pierce Biotechnologies, Waltham, MA) was dissolved in anhydrous DMSO and 5 μ L of a 4 mM solution was added to 50 μ L of the purified or recombinant protein solution and incubated at room temperature (RT) for 2 h. Thiolated-DNA (IDT) was resuspended to 100 μ M in Reaction Buffer and 3 μ L was added to 50 μ L of Reaction Buffer. To this solution, 4 μ L of a 100 mM solution of DTT (Life Technologies) was added to reduce the oxidized thiol-DNA. The solution was then incubated at 37 °C for 1 h. 7K MWCO (molecular weight cut-off) gel microspin columns (#89882, Thermo Fisher, Waltham, MA) were equilibrated to Reaction Buffer. The excess DTT and SMCC were removed by desalting with these equilibrated columns. The thiol-DNA and allergen-SMCC solutions were then mixed and reacted overnight at 4°C and then purified by 30k MWCO filter column (Millipore, Hayward, CA). Concentrations of the conjugates were measured by BCA assay (Life Technologies). Conjugation efficiencies were determined by SDS-PAGE and silver staining as described previously.⁶ Representative silver-stains are provided (Fig E1). DNA-to-allergen ratios of the conjugates were estimated by UV-VIS absorption. For each protein, the median number of conjugated DNA molecules is 2.5-3. Allergen-DNA conjugates were stored at 4°C for short-term usage or aliquoted for long-term storage at -80°C. Antibody-DNA conjugates were synthesized following a similar protocol, but with minor modifications. Briefly, anti-mouse IgE antibody was from Abcam (#ab19967). Anti-human IgE antibody was purchased from AbD Serotec (#STAR147). Instead of 30K MWCO filters, 100K MWCO filter columns were used to purify the conjugates from unreacted DNA.

Isotype-specific agglutination-PCR (ISAP). For detecting allergen-specific IgE against specific allergen components, 1 fmol of allergen-DNA conjugate and anti-IgE conjugates were suspended in 2 μ L of Buffer C (2% BSA, 0.2% Triton X-100, 8 mM EDTA in PBS). To this solution, 1 μ L of analyte was added and then incubated at 37°C for 30 min. After incubation, 117 μ L of ligation mix (20 mM Tris, 50 mM KCl, 20 mM MgCl₂, 20 mM DTT, 25 μ M NAD, 0.025 U/ μ L ligase, bridge oligo 100 nM, 0.001% BSA, pH=7.5) was added, and then incubated for 15 min at 30°C. After this incubation, 25 μ L of the solution was added to 25 μ L 2x PCR Master Mix (Thermo Fisher, Waltham, MA) with 10 nM primers and then amplified by PCR (95°C for 10 min, 56°C for 30s, 95°C for 15s, 13 cycles). The PCR reaction was then diluted 1:20 in ddH₂O and 8.5 μ L of the diluted PCR samples were added to 10 μ L 2x SYBR qPCR Master Mix (Life Technologies) with 1.5 μ L primers (final concentration 690 nM). Analysis by qPCR was performed on Bio-Rad CFX96 real-time PCR detection system. The overall process from sample input to assay completion was ~3-4 hours. Note that this method can be used to measure isotype-specific sIgE against specific allergen components; we have not attempted to produce versions for measuring sIgE against allergen extracts, which can contain a variety of different specific allergen components, often present in variable amounts in different extracts.^{7,8}

Comprehensive ligation-based immunoglobulin quantification (CLIQ). Our three-part PCR-based assay (CLIQ) is a combination of proximity ligation assay (PLA), antibody detection by agglutination-PCR (ADAP) and isotype-specific agglutination-PCR (ISAP). The combined

PCR-based assay detects total IgE, total anti-allergen antibodies and allergen specific IgE (sIgE) in a single assay. The protocol is similar to ISAP with minor modifications. For the comprehensive analysis of antibodies generated in response to OVA sensitization in OVA-immunized mice, 1 fmol of OVA-2A, OVA-2B, anti-IgE-1A and anti-IgE-1B were suspended in 2 μ L of Buffer C (2% BSA, 0.2% Triton X-100, 8 mM EDTA in PBS). To this solution, 1 μ L of analyte was added to the conjugates and then incubated at 37°C for 30 min. Following incubation, 117 μ L of ligation mix (20 mM Tris, 50 mM KCl, 20 mM MgCl₂, 20 mM DTT, 25 μ M NAD, 0.025 U/ μ L ligase, bridge oligo 100 nM, 0.001% BSA, pH=7.5) was added, and then incubated for 15 min at 30°C. Next, 25 μ L of the solution was added to 25 μ L 2x PCR Master Mix (Thermo Fisher, Waltham, MA) with 10 nM primers and then amplified by PCR (95°C for 10 min, 56°C for 30s, 95°C for 15s, 13 cycles).

Importantly, the multiplexed detection was achieved by PCR amplification with unique primer pairs (see Fig 1). For detection of total IgE, 1F and 1R primers were employed (shown in Fig 1 in dark blue and light blue); for total anti-OVA antibodies (of any isotype, including IgE), 2F and 2R primers were used (shown in Fig 1 in gold and yellow). For allergen specific IgE (IgE anti-OVA), 2F and 1R primers were used (shown in Fig 1 in gold and light blue). Each primer and PCR master mix were mixed with 25 μ L of ligation solution in separate wells. Then, the PCR reaction was diluted 1:20 in ddH₂O and 8.5 μ L of the diluted PCR samples were added to 10 μ L 2x SYBR qPCR Master Mix (Life Technologies, Carlsbad, CA) with 1.5 μ L primers (final concentration 690 nM). Again, total IgE was quantified by 1F and 1R primer pairs with qPCR (Bio-Rad CFX96 real-time PCR detection system). Total anti-allergen antibodies were quantified with qPCR by 2F and 2R primers. Specific IgE was quantified with 2F and 1R primers by qPCR. All analyses can be in different wells on the same qPCR plate for the same run.

To measure anti-peanut antibody responses in mice, a similar protocol was followed with few modifications. As probes, 1 fmol each of Ara-h1-2A, Ara-h1-2B, Ara-h2-3A, Ara-h2-3B, Ara-h3-4A, Ara-h3-4B, anti-IgE-1A and anti-IgE-1B were resuspended in 2 μ L of Buffer C (2% BSA, 0.2% Triton X-100, 8 mM EDTA in PBS) and 15 μ L of the solution was added to 15 μ L 2x PCR Master Mix with 10 nM primers. The primer pairs used in PCR and qPCR reaction included 1F/1R, 2F/2R, 2F/1R, 3F/3R, 3F/1R, 4F/4R and 4F/1R. The overall process from sample input to assay completion was ~3-4 hours.

For the detection of anti-peanut antibodies in patients' plasma, we performed procedures similar to those mentioned above. For those data points with disagreement between the two methods (i.e., the values fell within the false positive (FP), false negative (FN) quadrants), it remains to be shown, e.g., based on analyses of larger cohorts of patients, which of the two methods will yield results that are more concordant with clinical assessments (e.g., clinical history, physical examination, and, importantly, the results of oral food challenges). We also assessed the limit of detection for CLIQ, following the National Committee for Clinical Laboratory Standards (NCCLS) EP17-A protocol.^{9,10} Briefly, the limit of detection (LOD) is defined as the average of signals obtained with the blank specimen plus 3.29 standard deviations. Therefore, we calculated LODs of 0.95, 0.55 and 1.94 (Δ Ct

units) for anti-Ara h1, anti-Ara h2 and anti-Ara h3 sIgEs, respectively (indicated by the vertical red dashed-lines in Fig E9). As expected, the LODs are lower than the clinical cutoff values for each allergen-specific IgE.

Validation of ISAP with purified antibodies. Dilution series of anti-ovalbumin IgE (AbD Serotec #MCA2259, Hercules, CA), IgE isotype control (Biolegend #401701, San Diego, CA) and anti-ovalbumin IgG (Abcam #ab17293, Cambridge, United Kingdom) were prepared by performing 10-fold serial dilution of each antibody stock solution into Buffer C solution (2% BSA, 0.2% Triton X-100, 8 mM EDTA in PBS). The number of antibody molecules in each measurement was calculated by $(1 \times 10^{-6} \text{ L}) \times \text{Antibody concentration (M)}$. Therefore, the number of antibody in each dilution series ranged from 10^{-13} to 10^{-18} mol. Anti-IgE-1B and OVA-2A conjugates were used in these ISAP experiments. Primer 2F/1R were used for quantification. Data are shown in Fig 2A.

Validation of CLIQ with purified antibodies. Dilution series of anti-ovalbumin IgE, IgE isotype control and anti-ovalbumin IgG were prepared as described above. The number of antibody molecules also ranged from 10^{-13} to 10^{-18} mol. Anti-IgE-1A, Anti-IgE-1B, OVA-2A and OVA-2B were used in these CLIQ experiments. Primer pairs 1F/1R were used to quantify total IgE, primer pairs 2F/1R were used to quantify OVA-specific IgE, and primer pairs 2F/2R were used to quantify total anti-ovalbumin antibodies. Data are shown in Fig E2.

Data analysis. All PCR assays were run alongside a Buffer C-only blank (2% BSA, 0.2% Triton X-100, 8 mM EDTA in PBS) to correct for run-to-run variations. The Ct value for each sample was determined by a single-threshold fluorescence value automatically chosen by the Bio-Rad software. For each sample, the PCR cycle number with a fluorescence value corresponding to the threshold value was defined as the cycle threshold (Ct) value. ΔCt is defined as the Ct value of the blank minus the Ct value of the samples. The value of ΔCt is proportional to the initial amplicon concentration in the PCR plate well. This amplicon concentration is then also proportional to the amount of target antibody. To determine the detection limit, a non-linear four-parameter logistic fit for an antibody dilution series is determined using XLSTAT (New York, NY). The limit of detection for a PCR-based assay is defined as the average ΔCt value of the buffer C-only blank plus 3.29 standard deviations of the blank.^{9,10} Thus, the limit of detection value is calculated relative to the blank. A similar process was performed for dilution series of antibodies measured by ELISA to obtain the corresponding detection limit. For tests of specimens from mice undergoing OVA or peanut sensitization, we normalized the PCR-based signal to the signal observed at day 0.

Statistical analysis. For statistical analyses, unless otherwise specified, Mann-Whitney U tests were performed. We considered a *P* value of less than .05 to be statistically significant. Unless otherwise specified, data are shown as individual values or as mean $\pm$ S.E.M.

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FIGURE LEGENDS

Figure E1. Representative silver stains of allergen-DNA and antibody-DNA conjugates. DNA conjugated allergens or antibodies have a higher mass than the unconjugated counterparts. Thus, one can use gel analysis together with silver staining to confirm the success of the conjugation protocols. Lane 1: unconjugated Ara-h1. Lanes 2 and 3: Ara-h1-DNA conjugates. Lane 4: unconjugated Ara-h2. Lanes 5 and 6: Ara-h2-DNA conjugates. Lane 7: unconjugated antibodies. Lane 8: conjugated antibodies. The multiple bands observed in Lane 2, 3, 5, 6 and 8 are due to different numbers of DNA molecules conjugated onto the proteins. For each protein, the median number of conjugated DNA molecules is 2.5-3.

Figure E2. The integrated PCR-based Comprehensive Ligation-based Immunoglobulin Quantification (CLIQ) assay detects total IgE (tIgE) and allergen-specific (sIgE) with minimum cross-reactivity. We detected total IgE (tIgE, by proximity ligation assay, PLA), total anti-OVA specific immunoglobulins (total anti-OVA sIgE, by antibody detection by agglutination-PCR, ADAP) and anti-OVA specific IgE (anti-OVA sIgE, by isotype specific agglutination-PCR, ISAP) multiplexedly in a single assay (termed CLIQ, assay principles shown in **Fig 1D**). The specificity of the integrated CLIQ assay was assessed by testing separately dilution series of: **(A)** anti-OVA sIgG, **(B)** total IgE, and **(C)** anti-OVA sIgE, respectively. For instance, in **Figure E2, A**, the dilution series of anti-OVA sIgG was assayed by CLIQ. The PLA part of CLIQ measures total IgE and does not show signals (solid squares), the ADAP part of CLIQ, which measures total anti-OVA immunoglobulins, yields a strong concentration-dependent signal (solid circles), and the ISAP part of CLIQ, which measures anti-OVA sIgE, yields no signals (open triangles). For many of the data points (mean $\pm$ S.E.M), the error bars were too small to be seen.

Figure E3. Integrated PCR-based CLIQ analysis of serum and blood from OVA-sensitized mice. Serum was collected from OVA-sensitized and PBS-mock-sensitized control mice on days 0, 7, 14 and 21. The PCR signal was normalized to the day 0 value for each mouse. **(A)** Enhanced levels of total IgE [tIgE] were detected starting on day 7, anti-OVA sIgE on day 14 and total anti-OVA immunoglobulins on day 7. **(B)** ELISA analysis of anti-OVA sIgE in the same set of serum samples. Enhanced levels of anti-OVA sIgE were first observed on day 14. **(C)** Correlation between results of PCR-based analysis of total IgE using serum and whole blood samples from the same mice. Serum and whole blood values displayed a correlation coefficient (R) of 0.86. (In A-C, * represents $P < .05$.)

Figure E4. Integrated PCR-based CLIQ assay of whole blood of OVA-sensitized mice. In sensitized mice, PCR analysis of whole blood detected increased blood levels of total IgE [tIgE], anti-OVA sIgE, and total anti-OVA immunoglobulins on day 7. (* $P < .05$).

Figure E5. Body temperature after challenging peanut-sensitized mice with peanut extract or PBS. Peanut-sensitized BALB/c and C57BL/6 mice challenged with peanut exhibited a greater drop in body temperature than did identically-sensitized mice which were challenged with vehicle (PBS). (* $P < .05$ vs. values for the corresponding PBS-challenged BALB/c control mice, and + $P < .05$ vs. values for PBS-challenged C57BL/6 mice, by 2-WAY ANOVA).

Figure E6. Ara h 1 sIgE ELISA analysis of mouse serum. The ELISA procedure is described in the Methods section (following the protocol of Smit, et al.⁴). **(A)** Sera taken from mice before and after their oral sensitization to peanut (as described in Smit, et al.⁴ and Wijk, et al.⁵) were used as positive control specimens to validate the ELISA's ability to detect Ara h 1 sIgE. Briefly, 6 mg of peanut extract (#F171, Greer Laboratories, Lenoir, NC) with 15 μ g of cholera toxin (List Biological

Laboratories, Campbell, CA) per mouse were orally administered on days 0, 1, 2, 7, 14, 21, and 28. (n=3; five week-old female C3H/HeOuJ mice [Jackson Laboratories, Bar Harbor, ME]). Serum was collected on day 0 (Before) and day 30 (After). A statistically significant difference in levels of Ara h 1 sIgE was observed before and after peanut sensitization (* $P < 0.05$). **(B)** The sera shown in Figure 2D were re-analyzed by Ara h1 sIgE ELISA. No significant levels of Ara h 1 sIgE were detected.

Figure E7. Fold change in levels of total and allergen-specific IgE between day 0 (before sensitization) and day 42 (after sensitization), as measured by integrated PCR-based CLIQ assay of serum obtained from peanut-sensitized BALB/c and C57BL/6 mice (n=5 mice per strain). None of the differences in the results for the two strains of mice were statistically significant. The h1, h2, h3 labels in the x-axis refer to Ara h1, Ara h2 and Ara h3, respectively.

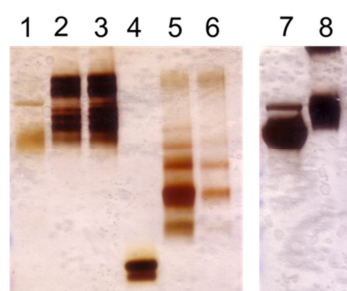
Figure E8. ELISA analysis of serum obtained from peanut-sensitized C57BL/6 mice (n=5 mice per strain) on day 0 (before sensitization) and day 42 (after sensitization). (These are the same serum samples analyzed by CLIQ assay in Fig E7.) No significant levels of anti-peanut specific IgE were detected.

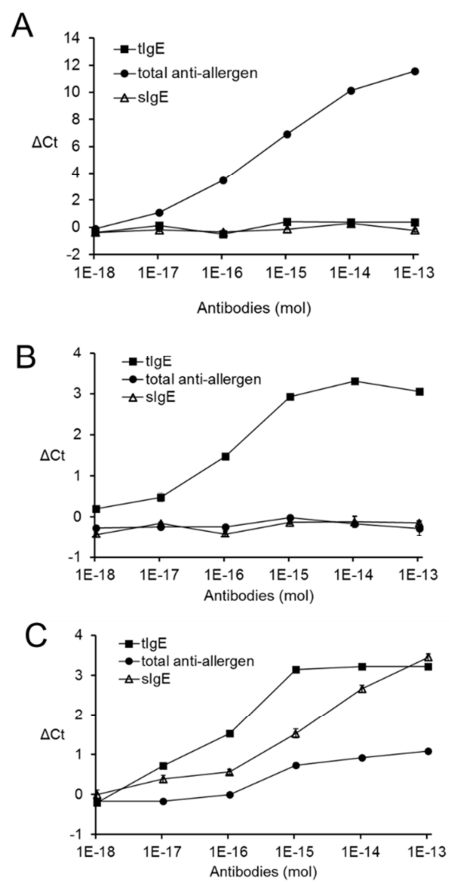
Figure E9. Comparison of results obtained by CLIQ versus ImmunoCAP. As described in Figure 2E, we plotted ISAP signals (ΔC_t) in the X-axis and the logarithm of ImmunoCAP signals (kU/L) in the Y-axis (the PCR readout ΔC_t is logarithmic by nature, therefore it was necessary to plot the logarithm of ImmunoCAP values for a valid comparison). In ImmunoCAP, 0.35 kU/L is the clinical cutoff for “sIgE positivity” (horizontal black dashed-lines). The CLIQ signals corresponding to 0.35 kU/L in ImmunoCAP are 1.11, 1.47, 1.99 (in ΔC_t units) for anti-Ara h1, anti-Ara h2, and anti-Ara h3 sIgE, respectively (vertical black dashed-lines). Then, as shown in the oval cartoon divided into 4 sections, we divided the correlation graphs into four quadrants: true positive (TP), false positive (FP), false negative (FN) and true negative (TN). Most data points fell within the TP (n=13, 16 or 12, in the left to right panels) and TN (n=5, 3 or 5, in the left to right panels) quadrants, thus confirming the high level of concordance between the results of the two methods. The overall agreements were 90%, 95% and 85% for anti-Ara h1, anti-Ara h2 and anti-Ara h3 sIgE, respectively.

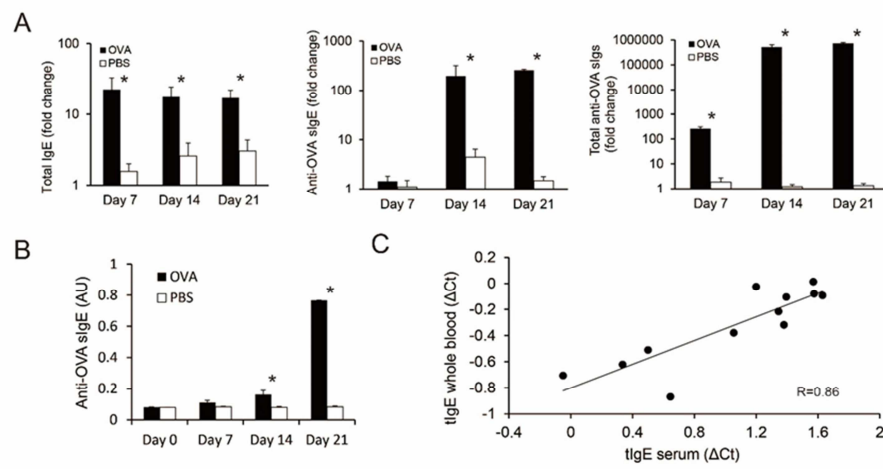
Figure E10. CLIQ assay clinical performance. To determine whether the assay cutoff established in Figure E9 is indeed clinically appropriate, we purchased 8 control serum samples of subjects not known to have peanut allergy from RDL Reference Laboratory (Los Angeles, CA). All samples were de-identified. We assayed these control serum samples with CLIQ. All control samples had sIgE levels against Ara h 1, Ara h 2 and Ara h 3 below the cutoffs.

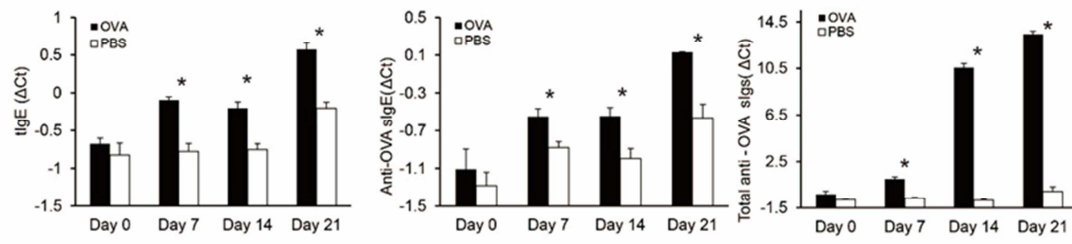
Figure E11. CLIQ assay reproducibility. We measured the same patient plasma specimens in 5 replicates in the same assay to determine the intra-assay variation, and also measured the same

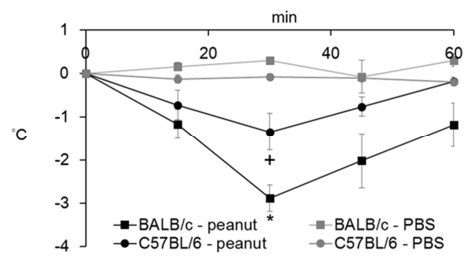
samples in 5 replicates on 4 different days to determine inter-assay variations. According to FDA guidance,¹¹ the generally acceptable intra-assay and inter-assay variations for bioanalytical methods are 15%.

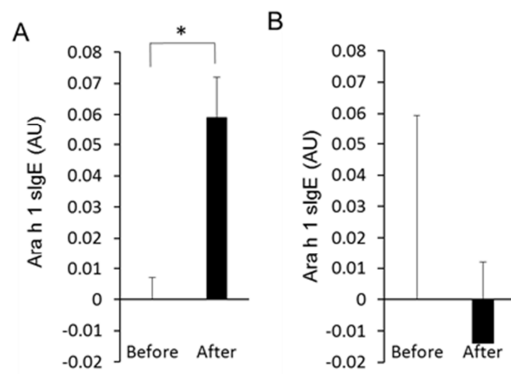


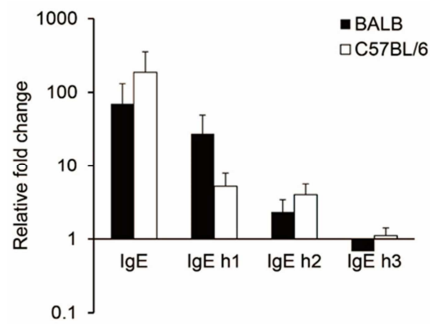


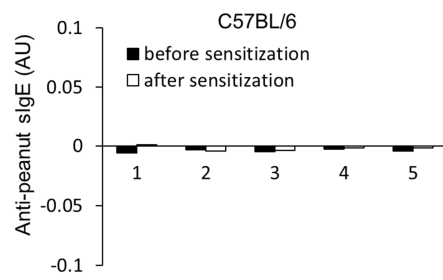


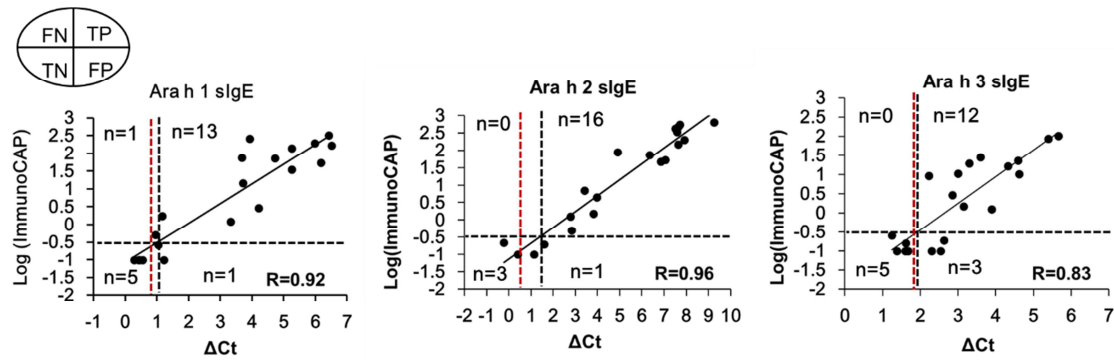


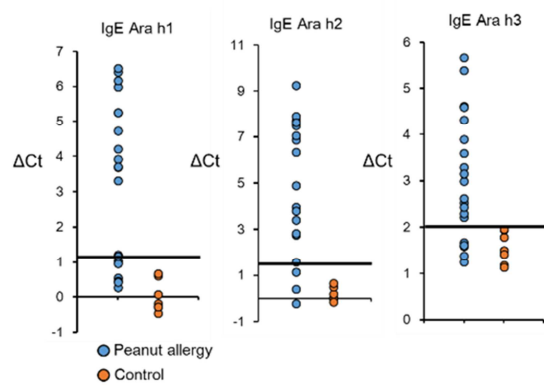












	Intra-assay	Inter-assay
Anti-Ara h 1 sIgE	10.9%	3.9%
Anti-Ara h 2 sIgE	6.6%	7.1%
Anti-Ara h 3 sIgE	4.7%	3.9%