Letter to the Editor

Isotype-specific agglutination-PCR (ISAP): A sensitive and multiplex method for measuring allergen-specific IgE

To the Editor:

Component-resolved diagnostics (CRD) is a rapidly growing class of in vitro tests for evaluating individuals thought to have allergic diseases. In contrast to using whole-allergen extracts, CRD uses individual, molecularly pure allergen components to measure allergen-specific IgE (sIgE), thereby helping to assess the risk of allergy to such allergens with improved accuracy.1 For example, the presence of sIgE against certain peanut components (eg, 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 Although ELISA-based CRD tests may lack analytical sensitivity, the alternative more sensitive assays currently used clinically (eg, ImmunoCAP) are not multiplexible and may omit certain critical allergens.2 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 multiplexable approach for measuring allergen-specific immunoglobulins in 1 μL of sample (Fig 1, A). ISAP uses 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 the 3′ portion of a split DNA barcode (Fig 1, A). Upon binding to the target immunoglobulin 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 reunits the 2 halves of the barcode to create a full-length DNA amplicon, which can then be quantified by quantitative PCR (qPCR). The amount of the amplicon directly reflects the quantity of analyte within the sample. Importantly, in the absence of the specified allergen-specific immunoglobulin, the 2 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-sIgE, we prepared a dilution series of (1) anti-ovalbumin (OVA) sIgE, (2) anti-OVA sIgG, and (3) total IgE (tIgE; control). The 3 samples were then analyzed with an ISAP assay designed to

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**FIG 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 2 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 tIgE. Samples are incubated with 2 anti–IgE-DNA conjugates that each bear half of a full-length DNA amplicon. C, ADAP for quantification of all antiallergen immunoglobulins (IgE, IgG, IgM, etc). This procedure is performed as before, but with 2 allergen-DNA conjugates. D, Integration of ISAP, PLA, and ADAP into a single assay, termed CLIQ. The sample is incubated with 2 allergen-DNA and 2 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. Ig, Immunoglobulin.
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 with ELISA (Fig 2, B).

In addition to sIgE, tIgE and total antiallergen specific immunoglobulin levels (of all immunoglobulin classes) are also of interest in characterizing allergic responses. To create an assay capable of measuring these parameters, we integrated ISAP with 2 other PCR-based methods: the proximity ligation assay (PLA)\(^5\) and antibody detection by agglutination-PCR (ADAP)\(^6\) (Fig 1, B and C). PLA uses 2 secondary antibody-DNA conjugates to detect tIgE, whereas ADAP uses 2 allergen-DNA conjugates to detect all allergen-specific immunoglobulins (eg, IgE, IgG, and IgM). The integrated assay, termed comprehensive ligation-based immunoglobulin quantification (CLIQ), converts sIgE, tIgE, and all specific immunoglobulins of any isotype into distinct DNA amplicons that can be independently interrogated with unique primer pairs (Fig 1, D).

To validate this concept, we reanalyzed the anti-OVA sIgE, anti-OVA sIgG, and tIgE (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 tIgE, 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 specific immunoglobulins, solid circles) shows concentration-dependent signals. The data in Fig E2 indicate that each of the 3 PCR-based methods selectively detected its specified target in an integrated assay without cross-reactivity.

Next, we sensitized mice with OVA1 and analyzed their antibody responses using CLIQ. CLIQ demonstrated elevations in serum and blood levels of IgE and total anti-OVA specific immunoglobulin at day 7 and sIgE at day 14 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 day 14 in the same OVA-sensitized mice (see Fig E3, B). 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; see Fig E6 in this article’s Online Repository at www.jacionline.org). 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 IgE and Ara h 1 sIgE after peanut oil sensitization in BALB/c or C57BL/6 mice (Fig 2, C; see Figs E7 and E8 in this article’s Online Repository at www.jacionline.org). In contrast, CLIQ did not detect such changes in antibody-deficient Jh-/- and Rag-/-/ mice after we attempted to induce peanut sensitization (Fig 2, C). These results show that the enhanced analytical sensitivity of CLIQ can detect potentially disease-relevant levels of sIgE that may be undetectable by traditional means such as ELISA.

Finally, we used CLIQ to analyze plasma obtained from 20 peanut-allergic subjects upon their enrollment into the institutional review board–approved peanut oral immunotherapy study; safety, efficacy and discovery trial (ClinicalTrials.gov Identifier: NCT02103270).9 CLIQ results displayed substantial positive correlations with ImmunoCAP data from the same specimens (Fig 2, E; 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 sIgE against specific allergen components in very small (1 µL) sample volumes. Integration of ISAP with PLA and ADAP to create the CLIQ assay greatly expands the information that 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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METHODS

Materials and reagents

Chemicals (including KCl, MgCl₂, Tris, EDTA, NAD, Triton X-100, and BSA) were purchased from Sigma-Aldrich (St Louis, Mo) and from Life Technologies (Carlsbad, Calif; including dimethyl sulfoxide, dithiothreitol [DTT], sulfosulfocoumarinimidyl 4-(N-maleimidomethyl)cyclohexane-1-carboxylic acid, and dNTP). All oligonucleotides were manufactured by Integrated DNA Technologies (abbreviated as IDT, Redwood City, Calif). The specific sequences for all oligonucleotides are provided in Table E1. Anti-OVA IgE was from AbD Serotec (#MCA2259, Hercules, Calif); IgE isotype control (also referred to as tIgE) was from Biologend (#401701, San Diego, Calif), and anti-OVA IgG was from Abcam (ab17293, Cambridge, UK). OVA protein was from Life Technologies (#77120) and Ara h 1 (#LTN-AH1-1), Ara h 2 (#RP-AH2-1), and Ara h 3 (#RNA-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 Epitope (#AS101, Madison, Wis), and DNA polymerase (#10966018) and SYBR qPCR 2X master mix (#STAR147) were from Thermo Fisher (Waltham, Mass). 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 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 the 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 in children and adults with peanut allergy (peanut oral immunotherapy study: safety, efficacy and discovery; ClinicalTrials.gov Identifier: NCT02103270). Peanut allergy was defined as having a reaction to a double-blind, placebo-controlled food challenge to peanut (with reactions occurring the mice by inhalation of CO₂).

Sensitization of mice was achieved by administration of OVA with alum. The protocol was performed as described in Balbino et al.\textsuperscript{11} with minor modifications. Briefly, mice (n = 5/group) were immunized intraperitoneally with 100 µg OVA (grade V; Sigma-Aldrich), 100 ng B pertussis toxin (List Biologicals, Campbell, Calif), and 50 µL Imject Alum (Thermo Scientific, Waltham, Mass) in PBS in a total volume of 100 µL on days 0 and 14. Control mice (n = 5) were inoculated similarly with 100 µL of a solution containing 100 ng B pertussis toxin and 50 µL Imject Alum in PBS, but without OVA. Serum and whole-blood samples were collected via retroorbital 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 1× 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<sup>−/−</sup> mice, and BALB/c-RAG<sup>−/−</sup> mice (all n = 5/group) was shaved and then depilated with Veet (Reckitt Benckiser, Slough, UK), followed by washing with PBS, and the mice were then sensitized once a week with 200 µL of peanut oil (#570600, Golden Peanut Company, Dawson, Ga) for 6 weeks. Per the manufacturer, the peanut oil is crude oil without any filtration or refinement, and Jablonski et al.\textsuperscript{12} confirmed the presence of peanut protein (~400 ppm) in this oil using colorimetric assays. After application, each mouse was gently held by the investigator for 1 minute for the peanut oil to be absorbed, and then 3 to 5 mice treated in this way were housed in the same cage. BALB/c and C57BL/6 mice were then challenged intraperitoneally with 5 mg of peanut protein (extracted from defatted peanut flour as previously described [Byrd Mill, Ashland, Va]).\textsuperscript{13} administered in 300 µL of PBS, or received PBS alone as a control. Rectal measurements of body temperature were taken 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 retroorbital bleeding and on day 42 via cardiac puncture after sacrificing the mice by inhalation of CO₂.

ELISA analysis

The ELISA to measure sIgE was performed as described in Balbino et al.\textsuperscript{11} 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. The streptavidin horseradish peroxidase conjugate reacts with its substrate 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 (#F717, 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-sIgE. To further measure Ara h 1 sIgE, we followed the protocol of Smit et al.\textsuperscript{14} and Van Wijk et al.\textsuperscript{15} Briefly, 96-well plates (Costar, Washington, DC) were coated with rat anti-mouse IgE (#553413, BD Biosciences, San Jose, Calif) at 4°C overnight. Following blocking with 5% BSA (Sigma-Aldrich) in PBS for 2 hours at room temperature, serum (1:8 dilution) was added for 2 hours at room temperature. Digoxin (DIG)-conjugated recombinant Ara h 1 (10 µg/mL, #LTN-AH1-1, Indoor Biotechnologies) was then added and incubated for 2 hours at room temperature. DIG was conjugated according to the manufacturer’s instructions (#55865, digoxigenin NHS-ester, Sigma-Aldrich). After incubation for an additional 2 hours 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

ImmunoCAP analysis was performed by Phadia, Thermo Fisher.

Synthesis of allergen-DNA and antibody-DNA conjugates

OVA was obtained from Life Technologies (#77120, Charlottesville, Va). Ara h 1 (#LTN-AH1-1), Ara h 2 (#RP-AH2-1), and Ara h 3 (#RNA-AH3-1) were purchased from Indoor Biotechnologies. Allergen (OVA, Ara h 1, Ara h 2, and Ara h 3)-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). Succinimidyl 4-(N-maleimidomethyl)cyclohexane-1-carboxylic acid (SMCC; Pierce Biotechnologies, Waltham, Mass) was dissolved in anhydrous dimethyl sulfoxide and 5 µL of a 4-mM solution was added to 50 µL of the purified or recombinant protein solution and incubated at room temperature for 2 hours. Thiocloted-DNA (IDT) was resuspended to 100 µM in reaction buffer and 3 µL was added to 50 µL of reaction buffer. To this solution, 4 µL of a 100-mM solution of DT (Life Technologies) was added to reduce the oxidized thiol-DNA. The solution was then incubated at 37°C for 1 hour. 7K Molecular weight cutoff (MWCO) gel microspin columns (#89882, Thermo Fisher) were equilibrated to reaction buffer. The excess DT 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, Calif). Concentrations of the conjugates were measured by BCA assay (Life Technologies). Conjugation efficiencies were determined by SDS-PAGE and silver staining as described previously.\textsuperscript{16} Representative silver stains are provided (Fig E1). DNA-to-allergen ratios of the conjugates were...
estimated by ultraviolet-visible spectrophotometry absorption. For each protein, the median number of conjugated DNA molecules is 2.5 to 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 300K MWCO filters, 100K MWCO filter columns were used to purify the conjugates from unreacted DNA.

**Isotype-specific agglutination-PCR**

For detecting allergen-specific IgE against specific allergen components, 1 fmol of allergen-DNA conjugate and anti-IgE conjugates was suspended in 2 μL of buffer C (2% BSA, 0.2% Triton X-100, 8 mM EDTA in PBS). To this solution, 1 μL of analyte was added and then incubated at 37°C for 30 minutes. After incubation, 117 μL of ligation mix (20 mM Tris, 50 mM KCl, 20 mM MgCl₂, 20 mM DTT, 25 μM NAD, 0.025 U/μL ligase, bridge oligo 100 nM, 0.001% BSA, pH 7.5) was added, and then incubated for 15 minutes at 30°C. After this incubation, 25 μL of the solution was added to 25 μL 2× PCR Master Mix (Thermo Fisher) with 10 nM primers and then amplified by PCR (95°C for 10 minutes, 56°C for 30 seconds, 95°C for 15 seconds, 13 cycles). The PCR reaction was then diluted 1:20 in ddH₂O and 8.5 μL of the diluted PCR sample was added to 10 μL 2× SYBR qPCR Master Mix (Life Technologies) with 1.5 μM 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 approximately 3 to 4 hours. Note that this method can be used to measure 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.

**Comprehensive ligation-based immunoglobulin quantification**

Our 3-part PCR-based assay (CLIQ) is a combination of PLA, ADAP, and ISAP. The combined PCR-based assay detects tIgE, total allergen antibodies, and allergen-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 each of OVA-2A, OVA-2B, anti-IgE-1A, and anti-IgE-1B was suspended in 2 μL of buffer C (2% BSA, 0.2% Triton X-100, 8 mM EDTA in PBS). To this solution, 1 μL of analyte was added to the conjugates and then incubated at 37°C for 30 minutes. Following incubation, 117 μL of ligation mix (20 mM Tris, 50 mM KCl, 20 mM MgCl₂, 20 mM DTT, 25 μM NAD, 0.025 U/μL ligase, bridge oligo 100 nM, 0.001% BSA, pH 7.5) was added, and then incubated for 15 minutes at 30°C. Next, 25 μL of the solution was added to 25 μL 2× PCR Master Mix (Thermo Fisher) with 10 nM primers and then amplified by PCR (95°C for 10 minutes, 56°C for 30 seconds, 95°C for 15 seconds, 13 cycles).

Importantly, the multiplexed detection was achieved by PCR amplification with unique primer pairs (see Fig 1). For detection of tIgE, 1F and 1R primers were used (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-sIgE (IgE anti-OVA), 2F and 1R primers were used (shown in Fig 1 in gold and light blue). Each primer and PCR master mix was mixed with 25 μL of ligation solution in separate wells. Then, the PCR reaction was diluted 1:20 in ddH₂O and 8.5 μL of the diluted PCR sample was added to 10 μL 2× SYBR qPCR Master Mix (Life Technologies) with 1.5 μM primers (final concentration 690 nM). Again, tIgE was quantified by 1F and 1R primer pairs with qPCR (Bio-Rad CFX96 real-time PCR detection system). Total allergen antibodies were quantified with qPCR by 2F and 2R primers. sIgE 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 h 1-2A, Ara h 1-2B, Ara h 2-3A, Ara h 2-3B, Ara h 3-4A, Ara h 3-4B, anti-IgE-1A, and anti-IgE-1B was resuspended in 2 μL of buffer C (2% BSA, 0.2% Triton X-100, 8 mM EDTA in PBS) and 15 μL of the solution was added to 15 μL 2× 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 approximately 3 to 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 2 methods (ie, the values fell within the false-positive, false-negative quadrants), it remains to be shown, for example, on the basis of analyses of larger cohorts of patients, which of the 2 methods will yield results that are more concordant with clinical assessments (eg, clinical history, physical examination, and, importantly, the results of oral food challenges). We also assessed the limit of detection (LOD) for CLIQ, following the National Committee for Clinical Laboratory Standards EP17-A protocol. Briefly, the LOD is defined as the average of signals obtained with the blank specimen plus 3.29 SDs. Therefore, we calculated LODs of 0.95, 0.55, and 1.94 (ΔCₜ units) for anti–Ara h 1, anti–Ara h 2, and anti–Ara h 3 sIgE, 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-sIgE.

**Validation of ISAP with purified antibodies**

Dilution series of anti-OVA IgE (AbD Serotec #MCA2259), IgE isotype control (Biolegend #401701), and anti-OVA IgG (Abcam #ab17293) 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 as (1 × 10⁻⁶ L) × Antibody concentration (M). Therefore, the number of antibodies in each dilution series ranged from 10⁻¹³ to 10⁻¹⁰ mol. Anti-IgE-1B and OVA-2A conjugates were used in these ISAP experiments. Primer pairs 2F/1R were used for quantification. Data are shown in Fig 2, A.

**Validation of CLIQ with purified antibodies**

Dilution series of anti-OVA IgE, IgE isotype control, and anti-OVA IgG were prepared as described above. The number of antibody molecules also ranged from 10⁻¹⁰ to 10⁻⁸ 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 tIgE, primer pairs 2F/1R were used to quantify OVA sIgE, and primer pairs 2F/2R were used to quantify total anti-OVA 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 CFX Manager Software (Bio-Rad, Hercules, Calif). For each sample, the PCR cycle number with a fluorescence value corresponding to the threshold value was defined as the 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 nonlinear 4-parameter logistic fit for an antibody dilution series is defined using XLSTAT (New York, NY). The LOD for a PCR-based assay is defined as the average of signals obtained with the blank specimen plus 3.29 SDs. Therefore, we calculated LODs of 0.95, 0.55, and 1.94 (ΔCₜ units) for anti–Ara h 1, anti–Ara h 2, and anti–Ara h 3 sIgE, respectively (indicated by the vertical red dashed lines in Fig E9).

Thus, the LOD value is calculated relative to the blank. A similar process was used 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 ± SEM.

REFERENCES


FIG 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 h 1. Lanes 2 and 3: Ara h 1-DNA conjugates. Lane 4: unconjugated Ara h 2. Lanes 5 and 6: Ara h 2-DNA conjugates. Lane 7: unconjugated antibodies. Lane 8: conjugated antibodies. The multiple bands observed in lanes 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 to 3.
The integrated PCR-based CLIQ assay detects tIgE and allergen-sIgE with minimum cross-reactivity. We detected tIgE by PLA, total anti-OVA specific immunoglobulins by ADAP, and anti-OVA sIgE by ISAP multiplexedly in a single assay (termed CLIQ, assay principles shown in Fig 1, D). The specificity of the integrated CLIQ assay was assessed by testing separately dilution series of (A) anti-OVA sIgG, (B) tIgE, and (C) anti-OVA sIgE, respectively. For instance, in Fig E2, A, the dilution series of anti-OVA sIgG was assayed by CLIQ. The PLA part of CLIQ measures tIgE 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 ± SEM), the error bars were too small to be seen.
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 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 tIgE using serum and whole-blood samples from the same mice. Serum and whole-blood values displayed a correlation coefficient (R) of 0.86. (In Fig E3, A-C, *P < .05.)
FIG 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 IgE, anti-OVA sIgE, and total anti-OVA immunoglobulins on day 7 (*P < .05).
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 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.)
FIG 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 al⁴). A, Sera taken from mice before and after their oral sensitization to peanut (as described in Smit et al⁴ and Wijk et al⁵) 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) with 15 μg of cholera toxin (List Biological Laboratories) per mouse was orally administered on days 0, 1, 2, 7, 14, 21, and 28. (n = 3; 5-week-old female C3H/HeOuJ mice [Jackson Laboratories, Bar Harbor, Maine]). 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 < .05). B, The sera shown in Fig 2, D, were reanalyzed by Ara h 1 sIgE ELISA. No significant levels of Ara h 1 sIgE were detected. AU, Arbitrary units.
FIG 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 2 strains of mice were statistically significant. The h1, h2, and h3 labels in the x-axis refer to Ara h 1, Ara h 2, and Ara h 3, respectively.
FIG 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 sIgE were detected. AU, Arbitrary units.
FIG E9. Comparison of results obtained by CLIQ versus ImmunoCAP. As described in Fig 2, E, we plotted ISAP signals ($\Delta C_t$) on the x-axis and the logarithm of ImmunoCAP signals (kU/L) on the y-axis (the PCR readout $\Delta C_t$ 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, and 1.99 (in $\Delta C_t$ units) for anti–Ara h 1, anti–Ara h 2, and anti–Ara h 3 sIgE, respectively (vertical black dashed lines). Then, as shown in the oval cartoon divided into 4 sections, we divided the correlation graphs into 4 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 2 methods. The overall agreements were 90%, 95%, and 85% for anti–Ara h 1, anti–Ara h 2, and anti–Ara h 3 sIgE, respectively.
To determine whether the assay cutoff established in Fig 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, Calif). All samples were deidentified. 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.

**FIG E10.** CLIQ assay clinical performance. To determine whether the assay cutoff established in Fig 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, Calif). All samples were deidentified. 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.
FIG E11. CLIQ assay reproducibility. We measured the same patient plasma specimens in 5 replicates in the same assay to determine the intraassay variation, and also measured the same samples in 5 replicates on 4 different days to determine interassay variations. According to Food and Drug Administration guidance, the generally acceptable intraassay and interassay variations for bioanalytical methods are 15%.

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<th>Intra-assay</th>
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<td>Anti-Ara h 3 sIgE</td>
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## Table E1. DNA sequence design

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