

## ORIGINAL PAPER

# Competition enzyme-linked immunosorbant assay (ELISA) can be a sensitive method for the specific detection of small quantities of allergen in a complex mixture

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## Clinical and Experimental Allergy

### Summary

**Rationale** The competition ELISA assay is used to determine the potency of US standardized allergen extracts. We have been concerned that the competition ELISA is not sensitive to changes in individual allergen levels. This study was designed to determine the sensitivity of the competition ELISA to detect the specific loss of Bla g 1 and Bla g 2 in cockroach extracts. **Methods** German cockroach extract E3Cg was made from defatted German cockroaches. New Zealand White rabbits were immunized with rBla g 1 or rBla g 2. Optimal dilutions of anti-Bla g 1 and anti-Bla g 2 sera were established by ELISA. E3Cg was selectively depleted of Bla g 1 or Bla g 2 by immunoabsorption with anti-Bla g 1 or anti-Bla g 2 attached to Protein G agarose beads. Competition ELISA using pooled human sera, or mixed anti-Bla g 1 and anti-Bla g 2 serum, was performed on the depleted extracts, and on depleted extracts reconstituted with rBla g 1 or rBla g 2.

**Results** Unlike pooled human-allergic IgE sera, anti-Bla g 1 and anti-Bla g 2 IgG – in dilutions as low as  $10^{-6}$ , could be used in the competition ELISA to measure the loss of allergen in depleted E3Cg. As little as 0.001 µg/mL of added rBla g 1 and 0.1 µg/mL of added rBla g 2, could be detected.

**Conclusion** The competition ELISA can be highly sensitive to compositional differences in complex allergen mixtures, even when the specific detecting antibody is present in relatively small amounts.

**Keywords** allergen, cockroach, ELISA, potency, standardization

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### Introduction

The potency of each manufactured lot of standardized allergen extract intended for sale in the US is estimated by comparison with a US reference standard. The potency measures, and the assays used to determine these measures, are specified in the approved product license applications of each manufacturer for each product; the choice of the best potency test depends on the allergen extract to be tested. While the tests for three allergen extracts (short ragweed, cat hair and cat pelt) focus on specific allergens of proven clinical relevance (Amb a 1 and Fel d 1, respectively), the potency of grass pollen and dust mite allergen extracts – for which the immunodominant components have not been determined with certainty – is determined by a test of overall allergenicity, the competition ELISA.

The competition ELISA is performed as follows. After coating the wells of the microtitre plate with the reference allergen, a mixture of the allergen extract to be tested and a reference IgE serum pool is added to the wells. The immobilized allergen competes with the test allergen added to the wells for free IgE antibody in the serum pool. The greater the amount of immunoreactive allergen in the mix, the less free IgE antibody will be available from the serum pool to bind to the immobilized allergen on the plate. As this assay does not explicitly measure a specific allergen, the allergen concentration is expressed as relative potency (RP) with the reference allergen extract assigned an arbitrary RP of 1.0 [1].

The competition ELISA has been demonstrated to be a good surrogate for the RAST inhibition assay [2] which, in turn, correlates well with *in vivo* potency determined by quantitative intradermal testing [3]. In experienced hands,

the competition ELISA is a remarkably consistent assay, with a standard deviation ( $\sigma$ ) of  $0.1375/\sqrt{n}$  in log RP units, where  $n$  is the number of replicate assays. This consistency has allowed manufacturers and regulators to control the safety and efficacy of these products with greater precision. In addition, it has permitted accurate comparison of potency with protein content [4] and quantitative assessments of manufacturing performance [5].

One concern about assays based upon overall potency is that they may be insensitive to changes in specific relevant allergens. In our study on the stability of dust mite allergenic extracts, we found a poor correlation between the loss of specific group 1 and group 2 allergens (as determined by monoclonal antibody-based two-site ELISAs), and the overall potencies of the extracts [6]. We have chosen to investigate further this aspect of the performance of the competition ELISA as part of our ongoing efforts to standardize German cockroach allergen extracts in the US. In the present study, we use mono-specific rabbit IgG antisera to deplete German cockroach allergen extracts of their Bla g 1 or Bla g 2 content. We then determine the ability to detect Bla g 1 and Bla g 2 in these extracts using competition ELISA assays performed with a human-allergic IgE serum pool, and pooled specific hyperimmune rabbit IgG sera.

## Methods

### Cockroach allergens

Cockroach extract E3Cg was produced from frozen whole *Blattella germanica*. Roaches were mechanically pulverized, defatted three times with cold ( $-18^{\circ}\text{C}$ ) acetone, air-dried and extracted with 10 mM ammonium acetate buffer (pH 8.5) containing leupeptin (12.5  $\mu\text{g}/\text{mL}$ ) and EDTA ( $1.25 \times 10^{-5}$  M). The extract was then filtered, frozen and lyophilized.

Recombinant (r) Bla g 1 or rBla g 2, both expressed in *Pichia pastoris*, were purchased from Indoor Biotech (Charlottesville, VA, USA).

### Immunization schedule

New Zealand white female rabbits were immunized monthly with three or four 50  $\mu\text{g}$  doses of rBla g 1 or rBla g 2. The first dose was prepared in complete Freund's adjuvant; subsequent doses were in incomplete Freund's adjuvant. Once acceptable levels of IgG antibody were attained, the rabbits were exsanguinated under anaesthesia.

### Electrophoresis and immunoblots

SDS-PAGE was performed under reducing conditions using Novex 4–20% Tris-glycine gels after denaturation in a sample buffer containing 0.0625 mol/L Tris (pH 6.8),

2% w/v SDS, 5% v/v  $\beta$ -mercaptoethanol and 10% v/v glycerol; separated proteins were visualized using a silver stain (Gel Code Silver Snap, Pierce, Rockford, IL). For the immunoblots, SDS-PAGE was performed using Novex 4–20% Tris-Bis gels, and the separated proteins were transferred to a polyvinylidene difluoride (PVDF) membrane (Invitrogen, Carlsbad, CA) in NuPage transfer buffer (Invitrogen) containing 20% methanol in an Invitrogen X Cell II Blot Module at 30 V for 2 h. The membranes were blocked with 5% non-fat dry milk in PBS containing Tween 20 (0.05%, v/v; PBS containing 0.1% Tween 20 (PBS-T)) for 1 h. They were incubated overnight at  $4^{\circ}\text{C}$  in anti-Bla g 1 or anti-Bla g 2 rabbit hyperimmune IgG serum ( $10^{-4}$  v/v in PBS-T), washed and placed in peroxidase-labelled goat anti-rabbit IgG (0.2  $\mu\text{g}/\text{mL}$  in PBS-T; KPL, Gaithersburg, MD, USA) for 2 h at room temperature. The membranes were then washed and developed with tetraethylbenzidine (TMB) membrane substrate (KPL, Gaithersburg, MD).

Direct ELISA was used to evaluate the antibody contents of the antisera used in the study. Microtitre wells were coated with 100 ng of rBla g 1 or rBla g 2. The wells were blocked and incubated with two-fold serial dilutions of serum. Bound rabbit IgG or human IgE was detected with biotin-labelled specific secondary antibody followed by peroxidase-labelled streptavidin and development with TMB substrate (all reagents from KPL). The control serum in this experiment was S5-grass, a human-allergic serum pool directed at grass pollen allergens.

Competition ELISA was performed as previously described [4, 7] with a reference pooled human-allergic IgE serum (S1Cr) or an optimized mixture of the rabbit hyperimmune IgG sera; when performed with the rabbit antisera, goat anti-rabbit IgG peroxidase conjugate was used instead of the anti-human IgE peroxidase conjugate. S1Cr was derived from the serum of 16 cockroach-allergic individuals [7]. In the competition ELISA, S1Cr was used at a dilution of  $10^{-1}$ . The optimized dilutions of the rabbit hyperimmune IgG sera for the detection of Bla g 1 depletion were  $2 \times 10^{-6}$  anti-Bla g 1 and  $5 \times 10^{-5}$  anti-Bla g 2. The optimized dilutions of hyperimmune IgG serum for the detection of Bla g 2 depletion were  $4.4 \times 10^{-7}$  anti-Bla g 1 and  $3.3 \times 10^{-5}$  anti-Bla g 2. In all cases the coating antigen and reference antigen was E3Cg, and the RP was determined by comparing the x intercepts of the parallel line inhibition curves [8].

### Depletion

The antigen depletion was performed using the batch method by gentle rotation at  $4^{\circ}\text{C}$ . Protein G Sepharose beads were washed with sodium acetate buffer (0.1 M, pH 5.0) and incubated with pre-immune rabbit serum at for 1 h. After washing with PBS pH 7.0, the E3Cg was incubated with the pre-immune serum beads for 1 h to

remove components of E3Cg that might bind non-specifically to agarose and rabbit serum proteins. The supernatant was then split into two equal aliquots. One was incubated for 2 h with fresh beads treated with pre-immune serum; the other aliquot was incubated for 2 h with fresh beads treated with rabbit hyperimmune serum (anti-Bla g 1 or anti-Bla g 2 IgG). At the end of the incubation, the supernatant was collected, mixed with glycerol to a concentration of 50% (v/v), and stored at  $-20^{\circ}\text{C}$ . In subsequent experiments, the supernatants incubated only with beads treated with pre-immune serum served as dilutional controls for the supernatants incubated with beads treated with hyperimmune sera.

Specific depletion was assayed by two-site ELISA performed at Indoor Biotech. In this assay, the mouse monoclonal capture antibodies were raised against native Bla g 1 or Bla g 2, the detection rabbit antisera were raised against the recombinant allergens, and the reference standards are native cockroach extracts the specific allergen contents of which had been previously established (<http://www.inbio.com/protocols.html>).

Repletion of the removed allergen was performed by adding rBla g 1 or rBla g 2 to the depleted extracts immediately before assay.

## Results

### Extract depletion

Using monospecific polyclonal rabbit IgG antisera, we were able to deplete the Bla g 1 and Bla g 2 content of German cockroach allergen extract E3Cg. The concentrations of Bla g 1 and Bla g 2 in the control extracts were 29 U/mL and 2.40  $\mu\text{g/mL}$ , respectively. Based upon the detection limits in these assays of 0.008 U/mL for Bla g 1 and 0.0015  $\mu\text{g/mL}$  for Bla g 2, the depletion efficiency was at least 99% for each allergen. The depletion was relatively selective: the loss of Bla g 1 in E3Cg<sup>dep 2</sup> was 30%, and the loss of Bla g 2 in E3Cg<sup>dep 1</sup> was 15% (Table 1).

Electrophoretically, evidence of depletion was subtle. Because of proteolytic cleavage of tandem amino acid

repeats, rBla g 1 from *P. pastoris* migrates at approximately four apparent molecular masses: 6, 21, 32 and 43 kDa [9]; the migration of native Bla g 1 is uncertain, but the same proteolytic cleavage likely occurs in the native molecule. Affinity-purified native Bla g 2 migrates at two apparent molecular masses, 36 and 70 kDa [10]. When stained with either Coomassie (not shown) or silver stain (Fig. 1a), the only band loss visible in E3Cg<sup>dep 1</sup> was below 10 kDa, presumably the 6 kDa proteolysis product, and there was no band loss in E3Cg<sup>dep 2</sup>. Immunoblots using the rabbit hyperimmune anti-Bla g 1 IgG serum likewise demonstrated the loss of only the most degraded Bla g 1 peptide, as well as a band at 15 kDa (Fig. 1b). Immunoblot using the rabbit hyperimmune anti-Bla g 2 IgG serum demonstrated loss of a single, faint band at 45 kDa (Fig. 1c), which may represent a partially degraded but still immunoreactive 70 kDa peptide.

### Competition ELISA using pooled human-allergic sera

Serum pool S1Cr contains human anti-cockroach IgE derived from 16 cockroach-allergic individuals, with sufficient anti-Bla g 1 and anti-Bla g 2 IgG to detect the presence of rBla g 1 and rBla g 2 in direct ELISA at serum dilutions of  $2.5\text{--}5.0 \times 10^{-2}$  (Fig. 2). In spite of this, S1Cr – when used in the competition ELISA at  $10^{-1}$  dilution – cannot detect the nearly complete loss of Bla g 1 or Bla g 2 in the depleted extracts (Figs 3a and b). Dilutional effects, equal in the allergen-depleted and sham-depleted extracts, resulted in 10-fold loss of RP when the depleted extracts were compared with untreated E3Cg.

### Competition ELISA with optimized anti-Bla g 1 and anti-Bla g 2 IgG

In contrast, the anti-Bla g 1 and anti-Bla g 2 rabbit IgG sera were able to distinguish between the allergen-depleted and sham-depleted extracts readily at serum dilutions as low as  $10^{-7}$  for anti-Bla g 1 (Fig. 4) and  $3.3 \times 10^{-5}$  for anti-Bla g 2 (not shown). As the failure of complex antibody mixtures such as S1Cr to detect specific antigen loss may be due, in part, to a poor signal-to-noise ratio, we attempted to construct mixtures of the two monospecific rabbit antisera to establish antibody ratios that could continue to detect allergen loss in spite of high signal from the other allergen still present. For the detection of Bla g 1 loss, the anti-Bla g 1 IgG serum could detect Bla g 1 depletion even at a  $2 \times 10^{-6}$  dilution and in the presence of a 25-fold excess of the anti-Bla g 2 IgG serum (Fig. 5a). However, the detection of Bla g 2 loss, while requiring the presence of only  $3.3 \times 10^{-5}$  dilution of anti-Bla g 2 IgG, was apparent only as long as the anti-Bla g 1 content of the detecting antibody mixture was kept relatively low, at 1.3% (v/v) of the anti-Bla g 2 IgG content (Fig. 5b).

Table 1. Specific depletion of Bla g 1 and Bla g 2 from E3Cg German cockroach allergen extract

|                         | Bla g 1 (U/mL) | Bla g 2 ( $\mu\text{g/mL}$ ) |
|-------------------------|----------------|------------------------------|
| E3Cg <sup>dep 1/c</sup> | 29             | 3.56                         |
| E3Cg <sup>dep 1</sup>   | Undetectable   | 3.02                         |
| E3Cg <sup>dep 2/c</sup> | 50             | 2.40                         |
| E3Cg <sup>dep 2</sup>   | 35             | Undetectable                 |

E3Cg<sup>dep 1</sup>, E3Cg depleted of Bla g 1 using anti-Bla g 1 IgG; E3Cg<sup>dep 1/c</sup>, control extract for E3Cg<sup>dep 1</sup>, processed using normal rabbit serum; E3Cg<sup>dep 2</sup>, E3Cg depleted of Bla g 2 using anti-Bla g 2 IgG; E3Cg<sup>dep 2/c</sup>, control extract for E3Cg<sup>dep 2</sup>, processed using normal rabbit serum. Assay detection limits, Bla g 1, 0.008 U/mL; Bla g 2, 0.0015  $\mu\text{g/mL}$ .

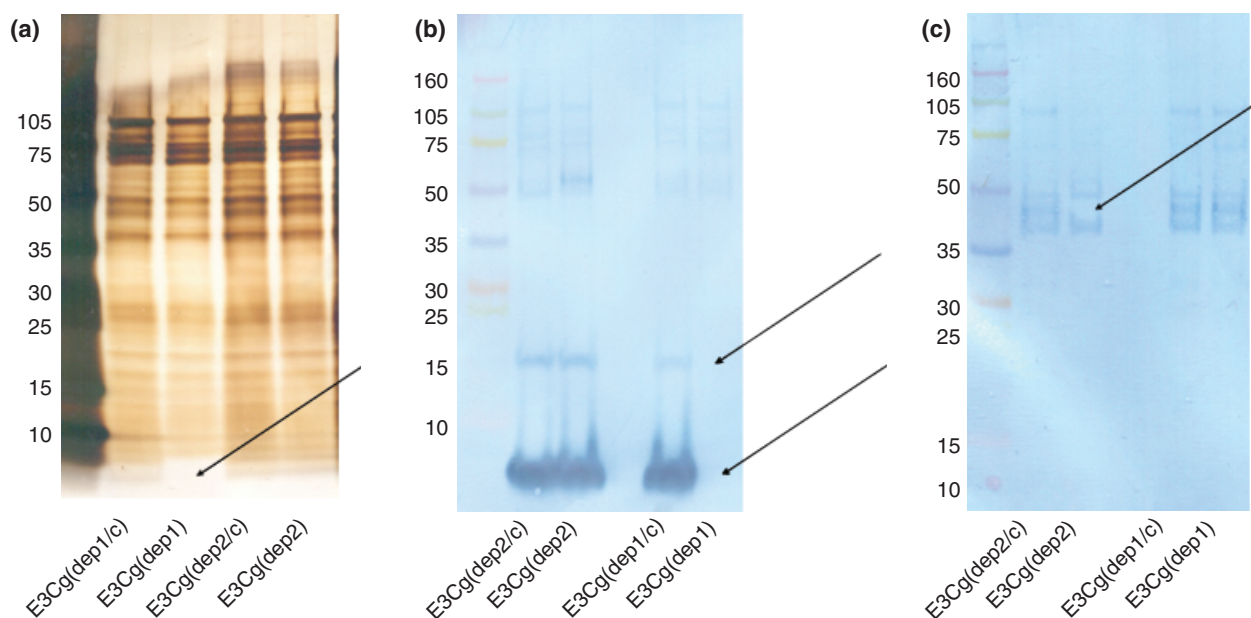


Fig. 1. SDS-PAGE and immunoblots of depleted extracts. Silver stained 4–20% SDS-PAGE (a) demonstrated the loss of a single band at  $< 10$  kDa in  $E3Cg^{dep1}$ , but no loss in  $E3Cg^{dep2}$ . Immunoblot developed with anti-Bla g 1 IgG (b) showed the loss of protein bands at 15 kDa and  $< 10$  kDa in  $E3Cg^{dep1}$ ; immunoblots developed with anti-Bla g 2 IgG (c) showed the loss of a protein band at 45 kDa in  $E3Cg^{dep2}$ . Abbreviations are as in Table 1.

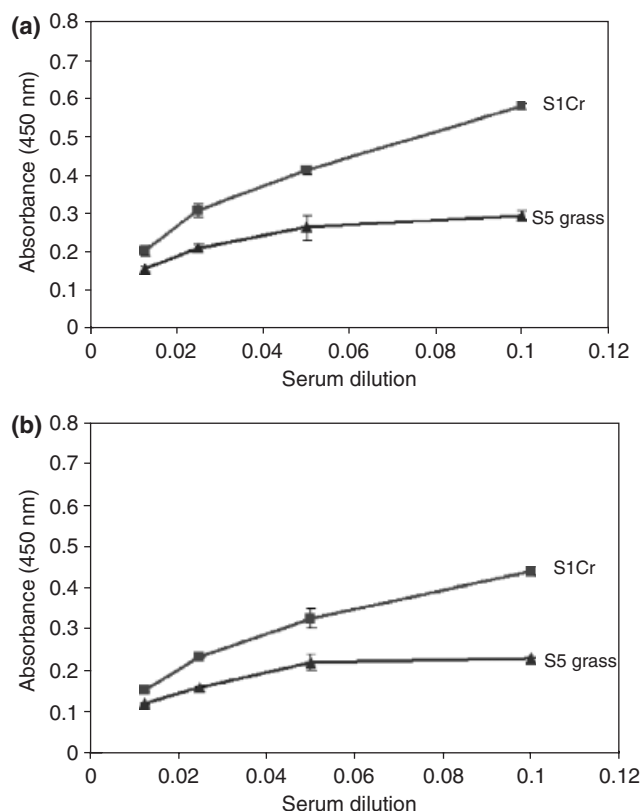


Fig. 2. Direct ELISA using pooled human serum (S1Cr) can detect rBla g 1 and rBla g 2 at serum dilutions as low as  $2.5\text{--}5.0 \times 10^{-2}$ . rBla g 1 (a) and rBla g 2 (b) were coated on microtitre wells and detected with either S1Cr or a negative control serum (S5-grass). Error bar = standard deviation of three replicates.

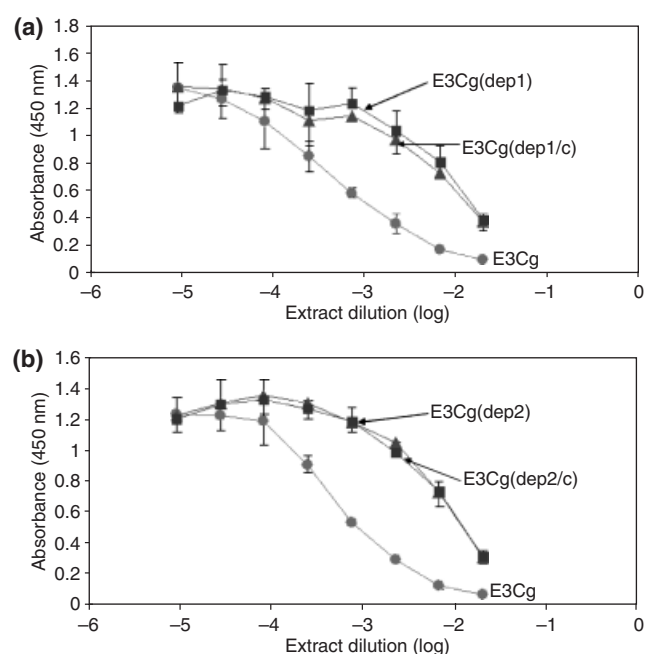


Fig. 3. Competition ELISA using pooled human serum (S1Cr) is unable to detect differences between allergen-depleted extracts and sham-depleted extracts. (a) Bla g 1 depletion. (b) Bla g 2 depletion. Abbreviations are as in Table 1. Error bar, standard deviation of three replicates.

#### Repletion of the depleted extracts

The sensitivity of the specific antisera to detect added recombinant allergen was assessed. Using the competition

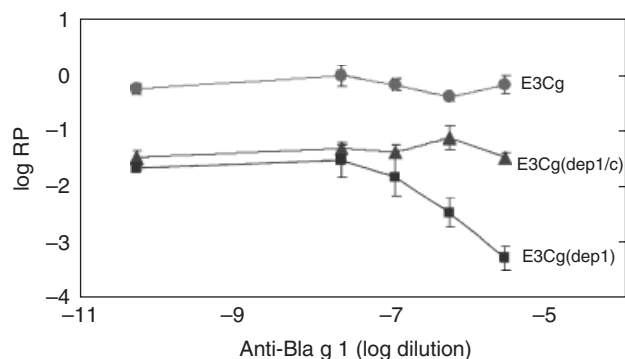


Fig. 4. Competition ELISA using anti-Bla g 1 serum at level detects loss of Bla g 1 in E3Cg<sup>dep 1</sup> at dilutions between  $10^{-5}$  and  $10^{-7}$ . Abbreviations are as in Table 1. Error bar, standard deviation of three replicates. The anti-Bla g 2 serum detected the loss of Bla g 2 in E3Cg<sup>dep 2</sup> at a dilution of  $3.3 \times 10^{-5}$  (not shown).

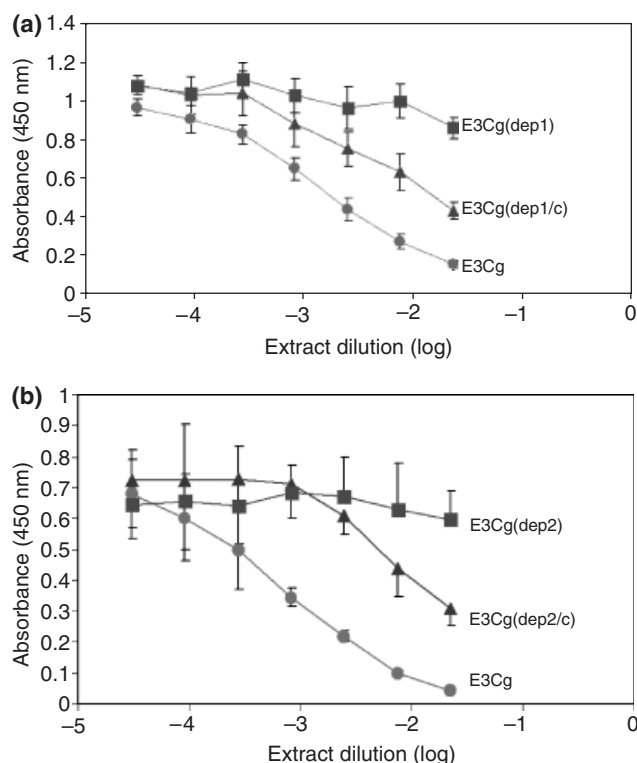


Fig. 5. Competition ELISA using an optimized mixture of anti-Bla g 1 and anti-Bla g 2 rabbit IgG serum detects differences between allergen-depleted extract and sham-depleted extract. (a) Bla g 1 depletion. (b) Bla g 2 depletion. Abbreviations are as in Table 1. Error bar, standard deviation of three replicates.

ELISA and the mixed rabbit antisera, we were able to detect as little as 0.001  $\mu\text{g/mL}$  of rBla g 1 added to E3Cg<sup>dep 1</sup>. The addition of 0.01  $\mu\text{g/mL}$  of rBla g 1 made the depleted extract indistinguishable from the undepleted control. Likewise, we were able to detect as little as 0.1  $\mu\text{g/mL}$  of rBla g 2 added to E3Cg<sup>dep 2</sup>. The addition of 0.2  $\mu\text{g/mL}$  of rBla g 2 made the depleted extract indistinguishable from the undepleted control (Fig. 6).

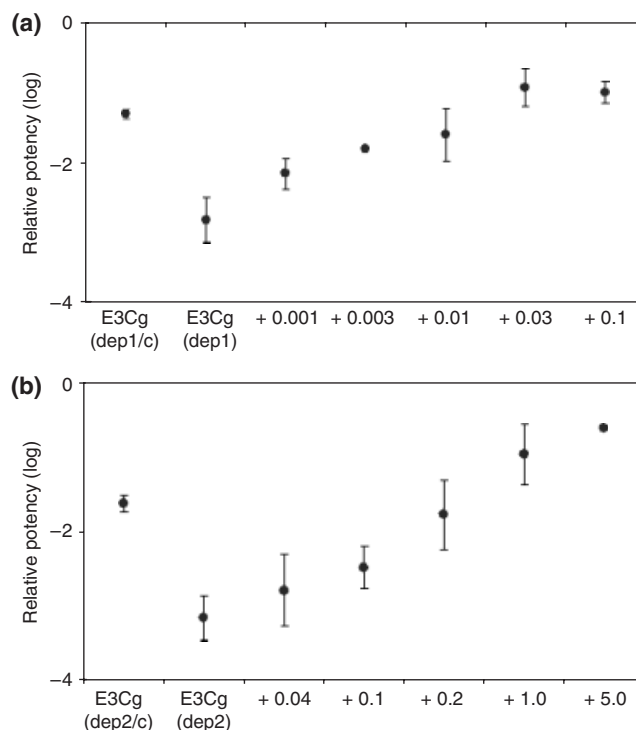


Fig. 6. Competition ELISA using optimized anti-Bla g 1/anti-Bla g 2 rabbit IgG serum detects the addition of specific allergens rBla g 1 (a) and rBla g 2 (b) to specific allergen-depleted extracts. Abbreviations are as in Table 1, with the numbers indicating the amount of specific allergen, in  $\mu\text{g/mL}$ , added to the depleted extract. Error bar, 95% confidence interval.

## Discussion

Tests for overall potency, such as the competition ELISA and the RAST inhibition test, are chosen to assess extract potency when the specific immunodominant allergens in an extract have not yet been identified. Such was the case when house dust mite and grass pollen extracts were standardized in the US, in the 1980s and 1990s, respectively. Thus, while other standardized extracts such as cat hair, cat pelt, and short ragweed are labelled with their specific allergen contents (Fel d 1 and Amb a 1), mite and grass pollen extracts are labelled in biological unitage assigned based on their overall RP. These potency tests are dependent upon the interactions of polyclonal and heterogeneous antibodies with a broad variety of allergenic molecules in the extracts, and the assay signal represents the integral of numerous such antibody/allergen interactions.

In our previous work with mite allergen extract stability [6], we found that the competition ELISA was insensitive to significant losses of specific group 1 and group 2 mite allergens. At that time, we hypothesized that all tests for overall allergenicity might be insensitive to changes in specific – and biologically significant – allergens. This

presented the undesirable possibility that such assays might become obsolete as specific immunodominant allergens are identified.

The present work was designed to address this question in the context of ongoing efforts to standardize German cockroach allergen extracts [7]. Our current understanding of cockroach allergy suggests that no one allergen is dominant in the US population, and that patterns of allergen recognition are highly variable [11]. Allergens Bla g 1, Bla g 2, Bla g 4 and Bla g 5 have all been suggested as important cockroach allergens [10–12]. Thus, while it is likely that no one specific allergen test will be the best assay for German cockroach allergen potency, any overall potency test should be capable of detecting a significant change in individual allergen levels.

Our efforts to deplete a crude cockroach extract of its Bla g 1 or Bla g 2 content were highly successful, with > 99% specific depletion. In spite of this, using the human serum pool S1Cr, we were unable to apply the competition ELISA to distinguish between our specific allergen-depleted cockroach extracts E3Cg<sup>dep</sup> 1 and E3Cg<sup>dep</sup> 2 and sham-depleted control extracts. However, when we used mixed rabbit hyperimmune sera, with antibody directed to the specific depleted allergens in dilutions of about  $10^{-6}$  to  $10^{-5}$ , we were able to detect the loss of specific allergen easily. Furthermore, using these sera, we could detect nanogram quantities of added recombinant-specific allergens.

Two possible reasons for the failure of S1Cr to detect the loss of specific allergen include low specific antibody titre and high noise generated by other, irrelevant antigen-antibody interactions. S1Cr was demonstrated to contain specific anti-Bla g 1 and anti-Bla g 2 IgE by direct ELISA, at dilutions two- to four-fold less than those used in the competition ELISA, so the signal should have been detectable. However, cockroach allergen extracts contain numerous allergens that are not detected by either anti-Bla g 1 or anti-Bla g 2 IgG (compare the SDS-PAGE to the immunoblots in Fig. 1), but are detected by the IgE in S1Cr (not shown). Thus, the noise generated by other antigen-antibody interactions is a likely explanation for our failure to detect specific allergen depletion using S1Cr. Furthermore, even with two hyperimmune rabbit sera of roughly equal titer, the detection of Bla g 2 in the allergen extract could be impaired by a relatively small amount of anti-Bla g 1 IgG in the detecting antibody mixture.

The major analytical issue attendant on these observations is that tests of overall potency, such as the competition ELISA, will be able to detect radical alterations in specific allergen levels only if they are specifically designed to do so. For cockroach allergen standardization, as for some other allergens already standardized, the challenge is to develop assays that balance our uncer-

tainty regarding the existence and identity of immunodominant allergens with evolving science on the identification of those allergens. This study demonstrates that, with proper reagents and careful design, the competition ELISA can be a reliable measure of overall potency as well as an accurate bellwether of specific allergen levels.

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