

# Convenient, rapid and economic detection and semi-quantification of American cockroach allergen in the environment

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## Summary

**Background:** Measuring allergen levels in the environment provides useful information to guide the management of allergic patients. A laboratory-based test kit sandwich ELISA for quantification of Per a 9, the major allergen of *Periplaneta americana* was recently developed. However, it is not suitable for screening.

**Objective:** To develop a simple, rapid, and economic format for semi-quantification of Per a 9 assay using dot-blot ELISA technique.

**Methods:** The efficacy of direct dot-blot ELISA and sandwich dot-blot ELISA was evaluated. Direct dot-blot ELISA was selected for further modification into 6 protocols. The selected protocol of direct dot-blot was further compared with the laboratory-based test kit, sandwich ELISA.

**Results:** The lowest detection limits in protocols no. 1-6 were 3.9, 15.6, 15.6, 62.5, 125 and 62.5 µg/ml of native Per a 9 whereas time required for each protocol was 145, 45, 30, 26, 18 and 26 minutes, respectively. The sensitivity of direct dot-ELISA was 3.9 µg/ml of Per a 9. Protocol no. 3 was the most suitable assay because its detection limits were as low as 15.6 µg/ml of CR allergen and the total process took only 30 minutes. In comparison with the 2 days required for laboratory sandwich ELISA, the selected protocol provided a similar yield of allergen detection but it offers significant savings of time. Additionally, this method could be easily interpreted by various groups of people.

**Conclusion:** This modified direct dot-blot ELISA is the first membrane ELISA which is a semi-quantitative test appropriate for screening American cockroach allergen owing to its simplicity, speed and good yield. (*Asian Pac J Allergy Immunol* 2012;30:99-106)

**Key words:** *Periplaneta americana*, American cockroach, Per a 9, arginine kinase, dot ELISA, allergen detection, environment

## Introduction

The prevalence and incidence of allergy caused by indoor allergens have increased worldwide. This is attributable partly to greater urbanization and a change in people's life styles.<sup>1,2</sup> Nowadays, more people work in crowded cities and live in small apartments close to their work places. The apartments are air-conditioned and/or closed during the owners' working hours; thus there is less fresh air ventilation. These conditions favor accumulation of dust and indoor allergens including house dust mite (HDM), cockroach (CR) and pet dander.<sup>3</sup> Therefore, people have a tendency to be exposed sensitization to allergens, especially during their prolonged residence in living rooms and bedrooms.<sup>4</sup>

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Upon re-exposure to a significant level of the allergen, allergen sensitized individuals usually develop allergic morbidity which may be as severe as atopic asthma especially in the case of CR allergy.<sup>5,6</sup> Allergen avoidance is one of the current measures for managing allergic morbidity in sensitized subjects (secondary intervention) and for preventing naive individuals from being sensitized (primary intervention). However, the practice has received low compliance by the general population, as well as allergic patients, as most people are not aware of the existence of the allergens in their environment.<sup>7</sup> Thus, demonstration of the presence of the allergens in house dust is one way of convincing them of the need for such measures.

The prevalence of CR allergy in Thailand ranges from 44-61% among atopic subjects.<sup>8,9</sup> American CR, *Periplaneta americana*, is the predominant source of CR allergens.<sup>10</sup> The major CR proteins that are bound to serum IgE of all CR allergic Thai patients include Per a 1<sup>11</sup> and Per a 9 (arginine kinase).<sup>12</sup> Thus, detection of the Per a 1 or Per a 9, or both, in the patient environment should be performed for implementation of the allergen avoidance measures. In 2003, two hybridoma clones secreting mouse monoclonal antibodies (MAb) specific to Per a 1 (clone 3C2) and arginine kinase (clone 38G6) were established.<sup>13</sup> In 2004, a sandwich (plate) ELISA (using MAb3C2 to Per a 1 as an allergen capture reagent and rabbit polyclonal antibodies (PAb) to American CR whole body extract (CR-E) as an allergen detection reagent was invented and used to assess Per a 1 distribution and quantification in the homes of CR allergic Thais. The highest concentration of the allergen was found in kitchens. Nevertheless, significant levels (threshold or higher levels for sensitization and morbidity) were detected in living rooms and bedrooms.<sup>10</sup> In 2009, a sandwich ELISA based on MAb38G6 to Per a 9 (arginine kinase) was used for determining seasonal variations of the CR allergen in dust samples collected from houses of CR allergic patients in the Bangkok metropolitan area.<sup>14</sup> The highest levels were found in houses made of wood and in the cooler months but significant levels for sensitization and morbidity were detected throughout the year. The MAb-PAb based sandwich ELISA for detecting and quantifying the Per a 1<sup>13</sup> and Per a 9<sup>14</sup> was sensitive and specific in measuring the levels of the respective allergens. Nevertheless, the assay is only suitable for the laboratory setting and was rather complicated and inappropriate for

screening. Thus in this study, a direct dot-ELISA which is a more simple, rapid, convenient and economic format for semi-quantification of Per a 9 has been developed and verified.

## Methods

### *Collection and extraction of house dust samples*

Twenty dust samples were collected from kitchens of CR allergic Thai patients by using a vacuum cleaner. Each sample was from an area of one square meter and the suction was operated for two minutes. The samples were extracted as described previously<sup>14</sup> and kept at -20 °C until use.

### *Monoclonal antibody (MAb) to Per a 9 (arginine kinase) of P. americana*

A hybridoma clone 38G6, which secretes mouse monoclonal antibody (MAb38G6) that binds specifically to the protein arginine kinase (Per a 9), a major allergen of American CR, has been established.<sup>13</sup> The immunoglobulin isotype of the MAb38G6 was IgG1/κ. For this study, the hybrid cells were grown in a serum-free medium (Gibco, Invitrogen Corp, Grand Island, NY, USA). Spent culture medium at the maximum phase of the cell growth was collected and the immunoglobulin was precipitated out from the culture medium using 50% ammonium sulfate. After being dialyzed thoroughly against cold 0.15 M phosphate buffered saline, pH 7.4 (PBS), the protein content and the indirect ELISA titer against *P. americana* CR-E of the MAb preparation were determined.<sup>13</sup> The MAb was used as a capture reagent in a sandwich dot-ELISA or as a detection reagent in a direct dot-ELISA for semi-quantification of the Per a 9 in dust samples collected from houses of CR allergic patients.

### *Preparation of rabbit polyclonal antibodies (PAb) to P. americana extract*

All animal experiments in the present study were approved by Siriraj Animal Care and Use Committee (SI-ACUP) no. 003/2554.

A New Zealand White rabbit weighing about 2 kg was immunized intramuscularly with five doses of the CR-E at 14 day intervals. The prime dose was 1 mg CR-E in 1 ml of PBS mixed with an equal volume of complete Freund's adjuvant. Each booster dose was 0.5 mg of the CR-E in 1 ml PBS mixed with equal volume of incomplete Freund's adjuvant. Two weeks after the last booster, the rabbit was bled, the immune serum was collected, total immunoglobulin was precipitated out by 50% ammonium sulfate and the polyclonal IgG (PAb)



was purified by using the protein-G affinity resin. The protein concentration of the PAb was measured<sup>15</sup> and adjusted to 1 mg/ml in PBS.

#### ***Preparation of native Per a 9 (arginine kinase) of P. americana***

Native arginine kinase was prepared from the CR-E of adult American CR by MAb38G6 based-affinity chromatography.<sup>12</sup> Briefly, CNBr-activated Sepharose™ 4B (GE Healthcare, NJ, USA) was mixed with purified MAb38G6 according to the instruction guide. The CR-E was blended with affinity gel on a rotating platform at 4°C overnight. After discarding the unbound fluid and washing with PBS, the bound Per a 9 was eluted out with 0.1 M glycine-HCl buffer, pH 3.0. One ml fractions were collected in tubes which each contained two drops of 1 M Tris-HCl (pH 8.0). After measuring the optical density (OD) at absorbance 280 nm (OD<sub>280nm</sub>), fractions containing protein were pooled and dialyzed against distilled water at 4°C overnight. This preparation was used as a reference Per a 9 (arginine kinase) in the ELISAs for (semi-) quantification of the Per a 9.

#### ***Dot-ELISAs***

Two versions of dot-ELISA, *i.e.*, sandwich- and direct- dot-ELISAs, were performed for semi-quantification of Per a 9 in CR-E and dust samples. For the sandwich dot-ELISA, 3 µl aliquots of MAb38G6 specific to Per a 9 were dotted onto centers of individual squares made on a nitrocellulose membrane (NC) and air-dried. Unoccupied sites on the NC were blocked with 3% bovine serum albumin (BSA) in PBS for 30 minutes. After the blocking reagent was removed by washing with PBS containing 0.05% Tween-20 (PBS-T), the MAb-dotted NC was allowed to react with various concentrations of Per a 9 ranging from 0.06 to 1,000 µg/ml PBS for 1 hour. The MAb dots which reacted to PBS served as negative allergen control. After washing with PBS-T, the NC was immersed into a solution of 1:1,000 rabbit immunoglobulin to the CR-E, kept for 1 hour and washed. Then, the NC was placed in a solution of goat anti-rabbit immunoglobulin-alkaline phosphatase (AP) conjugate (diluted 1:1,000) at 25°C for 1 hour. After washing, the membranes were put into the BCIP/NBT substrate solution for 5 minutes. The reaction was stopped by rinsing the NC with distilled water. Amounts of the Per a 9 and intensity of the color of individual spots (color levels) were observed visually.

For direct dot-ELISA, 3 µl of Per a 9 (various amounts) were dotted directly onto the NC squares and air-dried. After the empty sites on the NC were blocked with 3% BSA in PBS and washed as above, the NC was placed in MAb38G6 solution (2 µg/ml). After washing, the NC was submerged in rabbit anti-mouse immunoglobulin-AP conjugate (diluted 1:500) and washed. Color development was performed as described for the sandwich dot-ELISA. There were six protocols for the direct dot-ELISA, called protocols no. 1-6, depending upon the time allowed for each reaction step (Table 1). The color levels of the dot-ELISAs in relation to the amounts of purified native Per a 9 dotted onto the NC were graded (6 to 1) according to color intensity. Correlation of the color levels and grades were carried out by six different persons including two female scientists (aged 24 and 25), one male scientist (aged 35), one female secretary (aged 32), one male janitor (aged 49) and a female janitor (aged 53). These persons, designated interpreters no. 1-6, respectively, were asked to interpret the color levels of the direct dot-ELISA results performed on the dust samples.

#### ***MAb-PAb-based sandwich (plate) ELISA for quantification of P. americana Per a 9 (arginine kinase) in dust samples***

MAb-PAb based sandwich ELISA was used as a standard method for detection and quantification of Per a 9 in the environment.<sup>14</sup> The concentration of Per a 9 in each dust sample was quantified by the sandwich ELISA as described previously.<sup>14</sup> Briefly, each well of a 96-ELISA plate was filled with 4 µg of MAb38G6 in 100 µl of carbonate-bicarbonate buffer, pH 9.6 and kept at 4°C overnight. Fluid in each well was discarded and they were washed with PBS-T and blocked with 1% BSA in PBS-T. After removal of excess blocking reagent and washing, 100 µl of individual dust extracts were added to appropriate wells and the plate was kept at 37°C for one hour. After washing, 100 µl of rabbit PAb to CR-E was put into each well and the plate was kept at 37°C for one hour. Goat anti-rabbit IgG-HRP conjugate (100 µl of 1:1,000) and ABTS peroxidase substrate (KPL, USA) were used for color development. The enzymatic reaction was stopped with 50 µl of 4 NH<sub>2</sub>SO<sub>4</sub>. The OD<sub>492nm</sub> of the content in each well was measured against blank (the allergen/dust sample was replaced with PBS) with an ELISA reader (Multiscan EX; Lab systems, Helsinki, Finland). A series of known amounts of native Per a 9 were included in the same plate to



**Table 1.** Protocols no. 1-6 of the direct dot- ELISA.

Protocol no.	Time in minute(s) allowed for each reaction step						Total time allowed in minutes
	Spot arginine kinase (3 $\mu$ l)* onto NC squares and air-dried	Blocked with 3% BSA in PBS	NC pieces in MAb. solution <sup>#</sup>	NC pieces in rabbit anti-mouse Ig-AP conjugate**	NC pieces in BCIP/NBT substrate		
1	10	10	60	60	5	145	
2	10	10	10	10	5	45	
3	10	5	5	5	5	30	
4	10	1	5	5	5	26	
5	10	1	1	1	5	18	
6	10	1	5	5	5	26	

\*contained undiluted native Per a 9 (3.9  $\mu$ g/3  $\mu$ l); \*\*1:1,000 for protocols no. 1-4 and 1:500 for protocols no. 5 and 6. Ig, immunoglobulin; AP, alkaline phosphatase

# 1  $\mu$ g/ml for protocols no. 1-4 and 2  $\mu$ g/ml for protocols no.5 and 6.

create a standard curve. The concentration of Per a 9 in the dust samples was determined by using the read-out ELISA OD<sub>492nm</sub> against the standard curve.

### Statistical analysis

Minitab and SPSS version 18 were used for calculating of the medians and ranges of the allergen contents in the dust samples. Spearman's rank correlation test was used for comparing 1) the Per a 9 levels in dust samples determined by MAb-PAB-based sandwich ELISA and direct dot-ELISA and 2) the interpretation of direct dot-ELISA by scientist and non-scientist groups.

### Results

Sandwich and direct dot-ELISAs were concurrently performed on the native Per a 9. It was found that the former had too much color background in the negative allergen control which confounded the result interpretation (data not shown). Therefore, direct dot-ELISA was used for subsequent experiments.

The lowest concentrations of the CR-E that could be detected by using protocols no. 1-6 of the direct dot-ELISA were 3.9, 15.6, 15.6, 62.5, 125 and 62.5  $\mu$ g/ml, respectively (Figure 1). Although the detection limit of the protocol no. 1 was the least amount, the total time required for the assay completion was as long as 145 minutes. The least amount of CR-E detected by protocols no. 2 and 3 was 15.6  $\mu$ g/ml but the protocol no. 3 took a shorter time for completion (30 minutes; protocol no. 2 took 45 minutes). Protocols no. 4 and 6 required 26 minutes but the least detectable amount was 62.5

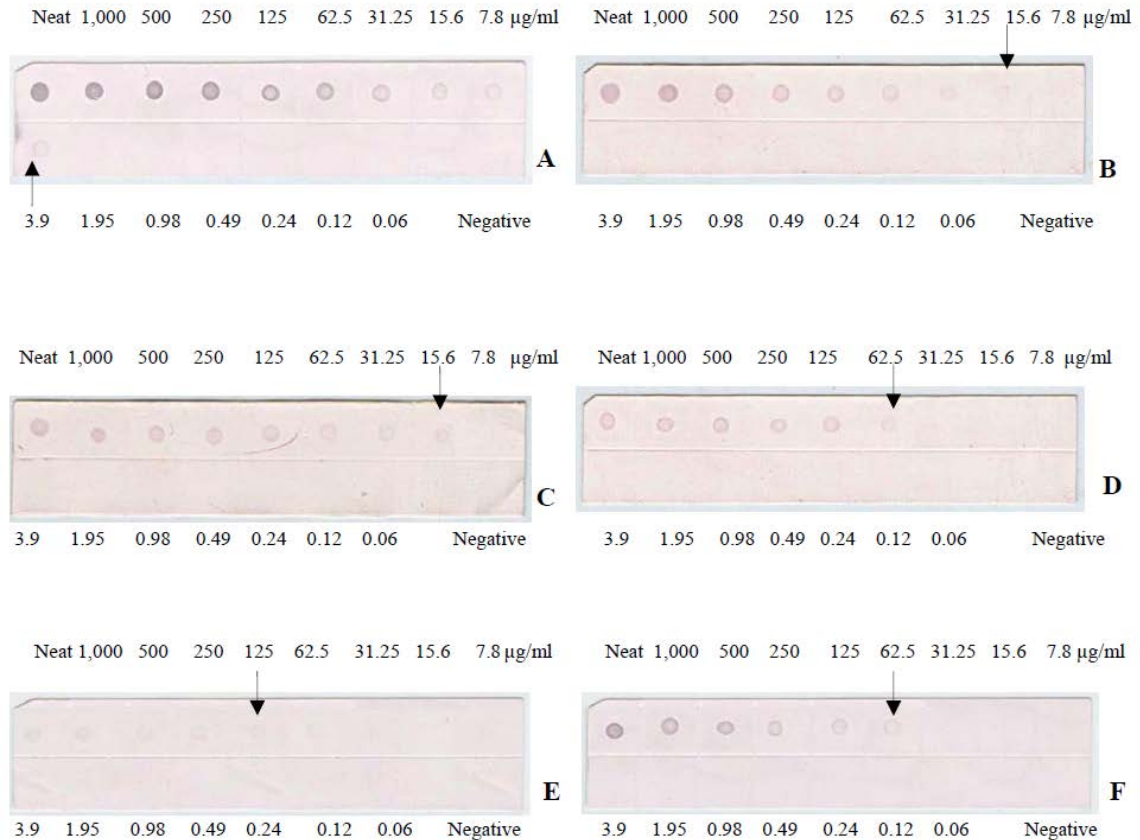
$\mu$ g/ml. Protocol No. 5 took 18 minutes but the detection limit was as high as 125  $\mu$ g/ml. Thus, protocol no. 3 of the direct dot-ELISA was chosen for detecting Per a 9 in dust samples in comparison to the quantification by laboratory based-sandwich (plate) ELISA.

The amounts of the CR-E, i.e., 250, 125, 62.5, 31.3, 15.6, 11.7, 7.8  $\mu$ g/ml correlated with the arbitrary color levels: 6, 5, 4, 3, 2, 1 and 0 of the protocol no. 3 direct dot-ELISA (Figure 2). These color levels and the designated grades shown in Figure 2 were used to instruct the six interpreters.

The color levels of all 20 dust samples tested by protocol no. 3 direct dot-ELISA are shown in Figure 3. The highest color level of the dust samples reported by the interpreters was 4 while the lowest level was 0.5.

The color levels of Per a 9 in all dust samples detected by protocol no. 3 direct dot-blot ELISA, interpreted by scientists and non-scientists, compared with the amounts of CR-E detected by the MAb38G6-PAB based sandwich ELISA are shown in Table 2. The color levels of dust samples tested by the direct dot-ELISA which were interpreted by three scientists were relatively similar and so were the interpretations by the three non-scientists ( $r = 0.96$ ). The correlation coefficients between the concentrations of CR-E in the 20 dust samples detected by the MAb-PAB-based sandwich ELISA and the median color-levels of the direct dot-ELISA interpreted by the six interpreters were calculated and are shown in Figure 4. There was a statistically significant correlation ( $p \leq 0.01$ ; two-tailed) between





**Figure 1.** The least amounts of native Per a 9 detected by different protocols of direct dot-ELISA. A-F, protocols no. 1-6, respectively. The detection limits of the six protocols were 3.9, 15.6, 15.6, 62.5, 125, 62.5 µg/ml, respectively. Neat, undiluted native Per a 9; numbers indicate Per a 9 concentrations in µg/ml (3 µl were dotted on to each spot). Negative, 3 µl of PBS.

CR-E levels detected by the sandwich ELISA and the median color-levels from the direct dot-ELISA interpreted by the three scientists (Figure 4A;  $r = 0.929$ ) and the color levels interpreted by the three non-scientists (Figure 4B;  $r = 0.893$ ). The results of direct dot-ELISA interpreted by both groups of interpreters and MAb-PAb based sandwich ELISA (Figure 4C) were also correlated statistically ( $r = 0.919$  at  $p \leq 0.01$ ).

**Discussion**

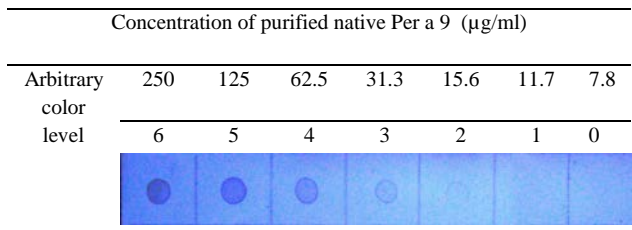
Monitoring indoor allergen levels in dwellings is an acceptable, valuable and effective intervention for preventing both the allergen sensitization and the development of allergic morbidity.<sup>16,17</sup> Currently, sandwich (plate) ELISA is used for measuring allergen in a sample.<sup>18,19</sup> Nevertheless, the assay has to be done in a laboratory and is relatively expensive and time consuming. There is a need for an allergen quantification assay that is simple, rapid and economic.

Thus in this study the previously invented sandwich ELISA (MAb-PAb based) was developed further into a direct dot-ELISA format. Considering to the cost, the NC utilized in dot-ELISA is cheaper than a 96-well microtiter plate ELISA employing in MAb-PAb based sandwich ELISA. The rabbit PAb to *P. americana* extract is essential in sandwich ELISA but not in dot-blot ELISA.<sup>14</sup> This PAb is not available commercially and the in-house preparation must be carried out by a skilled researcher using complicated techniques. Comparing the amount of utilized reagents, direct dot-ELISA requires only about one half of the volume that is needed in sandwich ELISA. Based on our estimation, the unit cost of dot-ELISA should be lower than that of sandwich ELISA. Hence, dot-ELISA is simple, does not require special equipment and consumes much less reagents, which make the test highly economic and cost effective. Moreover, the result is read visually.

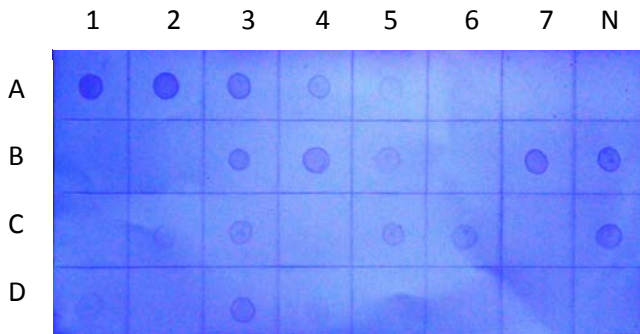


A comparison of two versions of dot-ELISA, i.e., sandwich- and direct-dot-ELISA revealed that the latter gave negligible background color which made result interpretation easier and gave a lower rate of false positivity. Besides, the direct dot-ELISA requires less reagent, less steps and less completion time.

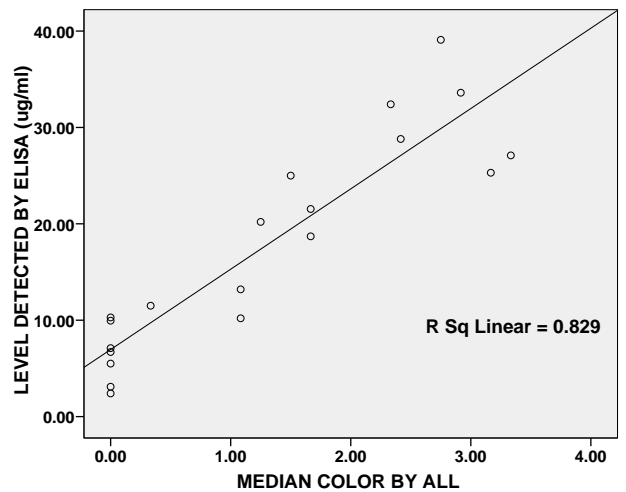
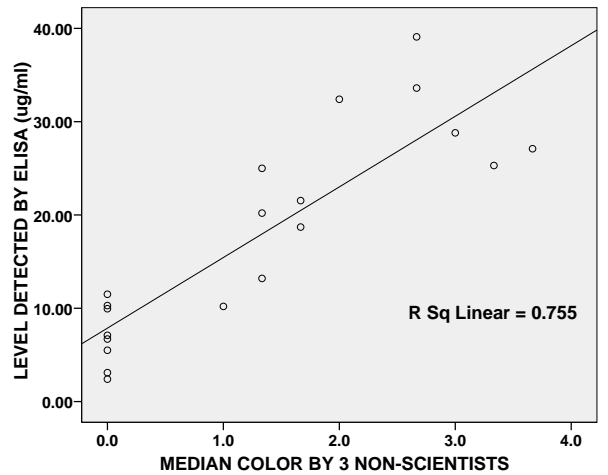
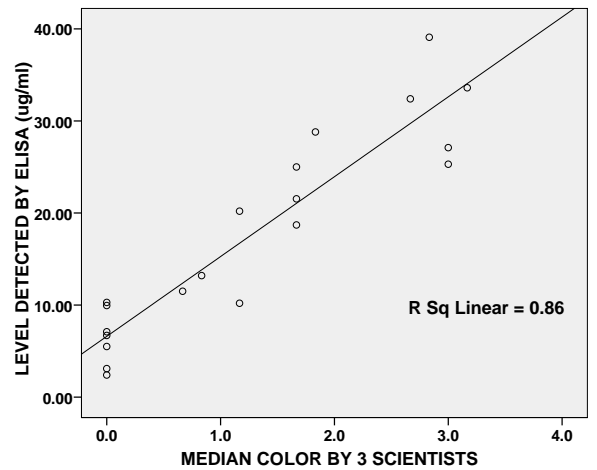
Six direct dot-ELISA protocols were validated to select the most appropriate one. The optimum time required for individual incubation steps were studied. With regard to the variation in the duration of each step that would affect the outcomes, two critical steps were the reaction between: 1) the dotted sample containing Per a 9 and the MAb38G6



**Figure 2.** Arbitrary color levels (range from 6 to 0) of the direct dot-ELISA spots dotted with various concentrations (250-7.8 µg/ml) of purified native Per a 9.



**Figure 3.** Results of the protocol no. 3 direct dot-ELISA for detecting known amounts of *P. americana* whole body extract in dust samples. Row A squares no. 1-7, dotted with 250, 125, 62.5, 31.3, 15.6, 11.7, 7.8 µg/ml of purified native Per a 9, respectively. The last square (N) was dotted with PBS as a negative control. Row B squares 1-8, house dust samples no. 1-8, respectively; Row C squares 1-7, dotted with samples 9-16, respectively; Row D squares 1-4, dotted with dust samples no 17-20, respectively.



**Figure 4.** Correlation of the allergen concentrations in dust samples detected by MAb-PAb-based sandwich ELISA and the median color-level determined by direct dot-ELISA. 4A, color levels of dot-ELISA interpreted by scientists ( $r = 0.929$ ); 4B, color levels of dot-ELISA interpreted by non-scientists ( $r = 0.893$ ); C, color levels of dot-ELISA interpreted by both scientists and non-scientists ( $r = 0.919$ ).

and 2) the allergen bound-MAb with the 1: 1,000 rabbit anti-mouse immunoglobulin-AP conjugate. The reaction time for these two steps should not be shorter than 5 minutes; the longer the time the better sensitivity, as seen in protocol no. 1 (time allowed was 60 minutes for each of the two critical steps; detection limit was 3.9 µg/ml) and the shortest time gave the least sensitivity, as seen in protocol no. 5 (detection limit was 125 µg/ml). The color intensity of the finished spots using to the protocol no. 5 (only one minute was allowed for each of the two critical steps) (Figure 1E) was much less than for the other protocols (Figure 1A, 1B, 1C, 1D and 1F). The detection limits of protocols no. 2 and 3 were the same, i.e., 15.6 µg/ml, but the time required for completion of the former (45 minutes) was longer than the latter (30 minutes) (Figure 1B and 1C, respectively). Thus, the protocol no. 3 was chosen.

Threshold levels for sensitization and asthmatic attack caused by *B. germanica* allergens were 2 and 8 U/g, respectively.<sup>7</sup> However, data are not available for the *P. americana* allergen. In a national survey, representative samples from 831 housing units inhabited by 2,456 individuals within 75 different locations throughout the US revealed that 63% of the households had Bla g 1 levels greater than the level required to trigger an asthmatic attack.<sup>20</sup> A survey made in Bangkok in 2004 revealed that the Per a 9 levels in dust samples collected from kitchens in houses of American CR allergic patients ranged from 4.40-82.00 µg per gram of dust and the median and mean allergen levels were high, i.e., 41.05 µg and 51.2 µg per gram of dust, respectively. A subsequent study in 2009, focusing on the seasonal variations of the amounts of Per a 9, revealed similar results. Although these data were from different geographical areas (USA and Thailand) and different kinds of CR allergens were reported in different units, it is apparent that CR allergic patients were exposed to high levels of CR allergens. Because the American CR allergen levels in the environment of the Thai CR allergic patients were higher than the detection limit of protocol no. 3 direct dot-ELISA, it is expected that the dot-ELISA can be used for monitoring the level of allergen in houses.

The finding that the color level interpretation of the direct dot-blot ELISA correlated with the amounts of Per a 9 tested by using the MAb-PAb based sandwich ELISA, shown in Table 2 and Figure 4 ( $r = 0.929/0.893/0.919$  at  $p \leq 0.01$ ) indicated that the efficacy of the direct dot-ELISA was

**Table 2.** Color levels of the direct dot-ELISA spots interpreted arbitrarily by six different interpreters and the amounts of CR-E in the samples tested by the sandwich (plate) ELISA.

Dust sample no.	Color level interpreted arbitrarily by interpreter no.						Level of CR-E detected by sandwich (plate) ELISA (µg/ml)
	1	2	3	4	5	6	
1	0	0	0	0	0	0	2.4
2	0	0	0	0	0	0	3.1
3	3	2.5	3	2	3	3	39.1
4	3.5	3	3	3	2	3	33.6
5	1	1	1.5	1	1	2	20.2
6	0	0	0	0	0	0	9.95
7	3	3	3	3	4	4	27.1
8	3	3	3	2	4	4	25.3
9	0	0	0	0	0	0	10.3
10	1	0.5	1	1	1	2	13.2
11	2	1	2	1	1	2	25.0
12	0	0	0	0	0	0	5.5
13	2	1	2	2	1	2	21.5
14	2	1	2	2	1	2	18.7
15	0	0	0	0	0	0	6.7
16	3	2	3	2	1	3	32.4
17	1	1	1.5	1	1	1	10.2
18	0	0	0	0	0	0	7.1
19	1.5	2	2	3	3	3	28.8
20	0.5	0.5	1	0	0	0	11.5

comparable to the laboratory based sandwich ELISA. In the direct dot-ELISA, the concentration of the Per a 9 could be estimated in relation to the intensity of color level; therefore the assay was considered to be a semi-quantitative method. In practice, the intensity of color levels for reference allergen could be set as: low, medium and high. The benefit is that the patients can have an idea of the level of allergen in their environment. Considering the practical use of the dot-blot assay, the ease of interpretation of the result by scientists and non-scientists was compared and revealed that the result from both groups was highly significant correlated ( $r = 0.96$ ). The correlation coefficient for the scientists (Figure 4A;  $r = 0.929$ ) was slightly higher than those from non-scientist group (Figure 4B;  $r = 0.893$ ), but there was no statistical significant difference between these two groups. This implies that this method could be used by anyone.

Compared with the laboratory-based test kit, the dot-blot assay has a number of advantages: it has

lower costs, it is easy to perform under field conditions in the absence of sophisticated resources and facilities and the results can be read directly by the naked eye. Moreover, the MAb-PAb based sandwich ELISA is considered to be a 2 day-process as it is necessary to coat the plate with MAb overnight one day before performing the test. Whereas, the developed dot-blot ELISA assay can be completed in a matter of hours. To our knowledge, this modified direct dot-blot ELISA is the first membrane ELISA which is convenient, rapid and economic and has the potential to become a viable semi-quantitative screening test kit for monitoring of American CR allergen in house dust.

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