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Corresponding Author:	ZhiFeng Huang, M.D Guangzhou Medical University Guangzhou, Guangdong CHINA
Corresponding Author's Institution:	Guangzhou Medical University
Corresponding Author E-Mail:	zfhuang_gzhmu@163.com
Order of Authors:	Zhifeng Huang Wenting Luo Xiaoqing Liu Xiangwei Zou Chuanxu Cai Zehong Wu Haisheng Hu Baoqing Sun, M.D
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TITLE:

Application of Biochip Microfluidic Technology to Detect Serum Allergen-specific Immunoglobulin E (IgE)

AUTHORS AND AFFILIATIONS:

Zhifeng Huang^{1,*}, Wenting Luo^{1,*}, Xiangwei Zou¹, Xiaoqing Liu¹, Chuanxu Cai¹, Zehong Wu¹, Haisheng Hu¹, Baoqing Sun¹

¹State Key Laboratory Respiratory Disease, National Clinical Research Center of Respiratory Disease, Guangzhou Institute of Respiratory Health, The First Affiliated Hospital of Guangzhou Medical University, Guangzhou Medical University, Guangzhou, China

*These authors contributed equally to this work.

Corresponding author:

Baoqing Sun (sunbaoqing@vip.163.com)

Tel: (86)-13922399896

Email addresses of co-authors:

Zhifeng Huang (zfh Huang_gzhmu@163.com)

Wenting Luo(348307079@qq.com)

Xiangwei Zou (394146317@qq.com)

Xiaoqing Liu (634431650@qq.com)

Chuanxu Cai (251483408@qq.com)

Zehong Wu (wuzehonghuzel@126.com)

Haisheng Hu (781640613@qq.com)

KEYWORDS:

biochip, microfluidics, BioIC, specific IgE, allergy, diagnosis

SUMMARY:

This paper presents a protocol for detecting serum-specific immunoglobulin E with a microfluidic cartridge-based chemiluminescence system and the evaluation of its usefulness in allergy diagnoses.

ABSTRACT:

Allergic disease is common in both adults and children. Identification of the causative allergens is significant in disease management and prevention. However, a specific immunoglobulin E (IgE) measurement system with a high price-performance ratio is lacking in mainland China, especially in the primary care hospitals. This paper describes the principle and operation procedures of using a microfluidic cartridge-based chemiluminescence system to detect allergen-specific IgE in serum. The results were compared with those from ImmunoCAP (System 1), the industrial standard, and the reproducibility of the system to detect patients sensitized to common allergens is evaluated. The results showed that in

comparison with ImmunoCAP (System 1), the BioIC System (System 2) has good precision and sensitivity in detecting serum-specific IgE against various inhalant and food allergens but with a significantly lower cost. It can serve as a good alternative to System 1 in primary care hospitals in mainland China who have lower financial affordability.

INTRODUCTION:

The prevalence of allergies has increased steadily in the past decades and is affecting 20%–30% of the global population¹. Identification of the causative allergens has important significance in managing the diseases. In China, since registered in vivo skin prick tests are not available in the country, in vitro determination of serum-specific IgE is the most important and commonly used tool for type I allergy diagnoses². This is similar to practices in the Western world, but although System 1, a fluorescence enzyme-linked immunosorbent base system, is perceived as the gold standard for in vitro allergy diagnosis³, its usage in China is very limited due to its high equipment and reagent price. Hence a new alternative allergy diagnosis system with a high price-performance ratio is badly needed.

The BioIC System (System 2) is a microfluidic cartridge-based system based on the chemiluminescence principle for multiplexed assays of serum-specific IgE. With a size of 7 cm x 4 cm, the microfluidic cartridge is composed of three layers of injection-molded plastic. The upper part is 3 mm-thick transparent polycarbonate which carries good stability during thermal assembly processes. Together with the 3 mm-thick bottom layer constructed from a copolymer of acrylonitrile, butadiene, and styrene (ABS), it sandwiches the 0.5 mm-thick middle layer made of silicone rubber. Being black in color, the middle layer offers lower background during chemiluminescence detection. On top of the silica gel, a thin layer of nitrocellulose membrane (NC membrane) is sprayed at the position corresponding to the reaction zone, which allows the spotting of different allergenic proteins. The purpose of this study is to evaluate the clinical performance of the microfluidic system for the multiplexed determination of allergen-specific IgE in serum.

PROTOCOL:

This study and the use of human serum samples were approved by the Ethics Committee of The First Affiliated Hospital of Guangzhou Medical University (GYYY-2016-73). All participants have given their written consent independently or through their parents (in the case of children).

1. Basic information of the study group

NOTE: The Allergy Information Repository of the State Key Laboratory of Respiratory Disease (AIR-SKLRD) is a large serum bank established inside the Guangzhou Institute of Respiratory Hospital (GIRH). Initiated in the last decade, the AIR-SKLRD has already started to collect and store serum samples from patients with allergic diseases, together with their clinical information (**Table 1**)^{4,5}. The current study was performed with sera from the AIR-SKLRD.

1.1. Search the AIR-SKLRD's database for sera collected from January 2015 to June 2018 and

select the patients with allergic disease, who were found to be sensitive to the common allergens in the region.

1.2. Ensure that all selected patients have allergic-related diseases, such as allergic rhinitis and or asthma, allergic dermatitis, and/or urticaria, and that the serum of these patients contains multiple serum allergen-specific immunoglobulin E (sIgE) sensitizations of common allergens in this region, detected by the System 1.

1.3. Exclude patients with incomplete medical records, those lost to follow-up, those who refuse to give informed consent regarding the use of their serum samples for scientific purposes, those with an identified immunodeficiency, those currently on immunotherapy or immunomodulatory agents, or those found to have parasitic infections.

1.4. Ensure no treatment or drug prescription was given prior to the serum collection so as to minimize interference to the laboratory findings. All serum samples that do not fulfill the criteria were rejected.

2. Study flow and measurements of interest

NOTE: The microfluidic system needs 100 μ L of serum for determining 19 allergens. Venous blood (5 mL) was collected from each patient using a vacuum blood vessel containing separating gel. After centrifuging for 10 min at 1,000 $\times g$, the upper layer was collected for testing. Unused serum was stored at -80 $^{\circ}$ C. Prior to testing, the serum was kept at room temperature for 30 min and was shaken with a vortex mixer. Repeated freeze-and-thaw cycles were avoided.

2.1. Primarily test the serum samples for sIgEs to whole allergens of *Dermatophagoides pteronyssinus* (d1), *Dermatophagoides farinae* (d2), *Blomia tropicalis* (d201), cat dander (e1), dog dander (e5), Bermuda grass (g2), timothy grass (g6), cockroaches (i6), *Aspergillus fumigatus* (m3), *Candida albicans* (m5), ragweed (w1), egg white (f1), milk (f2), wheat (f4), peanut (f13), soybean (f14), almond (f20), crab (f23), and shrimp (f24). Follow the instructions given in section 3.

NOTE: sIgE determination was done with the allergen-specific IgE assay kit (see the **Table of Materials**) and measured by a chemiluminescence analyzer.

2.2. Randomly select three samples from among the samples with enough serum (at least 900 μ L) for a reproducibility study. Keeping all the conditions unchanged, measure the three sera for allergen sIgEs daily for 9 consecutive days (i.e., a total of 100 \times 9 = 900 μ L of serum).

3. Semi-automation test procedure of the microfluidic system

NOTE: The System 2 is the integration of automatic microfluidic technology, protein microarray, cold light analysis, parallel IgE analysis, and image processing technology. The testing protocol

is divided into four parts: preparation of the equipment, sample loading, incubation, and measurement.

3.1. Preparation of the equipment

3.1.1. Turn on the PC and the analyzer power.

NOTE: The power switch is on the left of the base.

3.1.2. Start the LabIT program on the PC. If the **Dark Frame** warning window pops up, click **OK** to run **LEAK Test**. Afterward, click the center logo to enter the operation interface.

NOTE: The system will remind the user to run **LEAK Test** if it is idle for more than 24 h.

3.1.3. Check the Reaction Temp and CCD (charge-coupled device) Temp at the lower right corner of the screen. The Reaction Temp should rise to $37\text{ }^{\circ}\text{C} \pm 1\text{ }^{\circ}\text{C}$ in about 10 min, and the CCD Temp should drop to $-15\text{ }^{\circ}\text{C} \pm 1\text{ }^{\circ}\text{C}$.

3.1.4. Run the **LEAK Test** after the CCD Temp has dropped to $-15\text{ }^{\circ}\text{C} \pm 1\text{ }^{\circ}\text{C}$. Before running the LEAK test, make sure there are no other items left inside the instrument and close the door. Click **Tools | System Test | LEAK Test**. Do not open the door during testing. When the test is finished, the report window will pop up.

3.2. Sample loading

3.2.1. Add 620 μL of wash buffer, 120 μL of blocking buffer, 60 μL of conjugates A and B, 60 μL of substrate A and B, and 100 μL of serum samples to the corresponding reagent tank on the microfluidic cartridge.

3.3. Incubation

3.3.1. Click on **Cartridge ID**, use the barcode scanner to scan the serial number of the cartridge, enter the sample ID, put the cartridge into the analyzer and close the door, and click **Analyzer** and **Run** to start the analysis.

3.4. Measurement

3.4.1. Export the results to statistical software (e.g., Excel) after the measurement.

NOTE: After 30 min of incubation, the analyzer automatically performs the measurement and reports the result.

3.5. Switching off of the analyzer

3.5.1. For routine maintenance, after finishing the test, remove the cartridge and wipe the analyzer's internal heating iron and electromagnet lightly with 75% alcohol.

NOTE: Do not press hard or shake the electromagnet.

3.5.2. Close the LabIT window. The temperature monitoring window will pop up. It will automatically close when the CCD warms up to the 5 °C protection mode. By then, it will be safe to turn off the power of the analyzer and PC.

NOTE: Do not manually close the temperature monitoring window before the CCD Temp has risen to 5 °C, and do not turn off the power of the analyzer nor the PC during the CCD warm-up.

4. Definition of sIgE reactivity

NOTE: For an undiluted serum specimen, the detection range of the System 2 is 0.21–100 IU/mL.

4.1. Based on the threshold value of 0.35 IU/mL, consider an sIgE level exceeding 0.35 IU/mL to be positive^{6,7}. Rate the reactivity of the sIgE tests as⁸: class 1 (≥ 0.35 and < 0.70 IU/mL), class 2 (≥ 0.70 and < 3.50 IU/mL), class 3 (≥ 3.50 and < 17.50 IU/mL), class 4 (≥ 17.50 and < 50.00 IU/mL), class 5 (≥ 50.00 and < 100.00 IU/mL), and class 6 (≥ 100.00 IU/mL).

5. Statistical analysis

5.1. Use a histogram to show the positive rate of the 19 allergens (**Figure 1**) and use the Levey-Jennings curve to demonstrate the repeatability of the detection system (**Figure 2**)⁹.

5.2. Select the three most common inhalant allergens and food allergens (in total, six allergens) and compare the results to the I System 1 to evaluate its clinical diagnostic performance^{10,11}. Include the concordance rate, sensitivity, specificity, positive and negative predictive values, and the area under the receiver operating characteristic (ROC) curve (AUC) as the evaluation criteria.

5.3. Apply Spearman's correlation analysis¹² to describe correlations between the two systems and use kappa value for consistency. Categorize the kappa value as almost perfect (0.8–1.0), substantial (0.6–0.8), moderate (0.4–0.6), fair (0.2–0.4), or poor (< 0.2)¹³. Use SPSS 23.0 and MedCalc 11.0 statistical analysis and define $P < 0.05$ as statistical significance.

REPRESENTATIVE RESULTS:

Positive rates for 19 common allergens

The results on 293 sera are shown in **Figure 3**. Among all the inhalant allergens, *D. farinae* had the highest positive rate (80.89%, 273/293), followed by *D. pteronyssinus* (78.84%, 231/293).

Among the food allergens, crab has the highest positive rate (20.48%, 60/293), followed by 13.65% (40/293) for shrimp. The total positive rate for inhalant allergens was higher than that for food allergens.

Repeatability of the microfluidic system

Repeatability results for cat dander, dog dander, and cockroach, based on nine rounds of testing, were 32.98 ± 8.94 , 1.61 ± 0.48 , and 0.76 ± 0.18 , respectively, and the consistency levels were 100% (9/9), 100% (9/9), and 67% (6/9). Distribution of the results is shown with the Levey-Jennings curve in **Figure 2**. All data are within the range of $X \pm 2 \times SD$, which is consistent with the maximum allowable clinical error¹⁴.

Comparison of two systems

Qualitative results showed that cat dander had the highest concordance to System 1 (95.33%, 243/150). The lowest concordance was seen in shrimp (40.75%, 88/216). The total concordance among inhalant allergens ranged from 92.00% to 95.33%. For food allergens, the concordance range was 40.74%–72.39%. The highest sensitivity for inhalants was seen in *Dermatophagoides farinae* (93.94%), with a 100% specificity. Among food allergens, the highest sensitivity was seen in peanut (54.55%), with a specificity of 80.65%. **Table 2** also shows that all the evaluation results for inhalant allergens were superior to food allergens. Since the AUC values showed a range from 0.613 to 0.984 and the AUC for the three inhalant allergens was greater than 0.950, it can be concluded that the System 2 has a high accuracy with reference to System 1.

Consistency analysis for the two systems showed that the kappa values for the three inhalants were between ~ 0.727 – 0.876 , with the highest value seen in cat dander as 0.876 (95% CI, 0.0.786–0.965). They were all better than the kappa values for food allergens which, in general, fell < 0.400 . The lowest kappa value was 0.112 in shrimp (95% CI, 0.062–0.162) (**Table 3**). Spearman's correlation analysis showed that the best correlation was seen in peanut and cat dander, with correlation coefficients as $r = 0.942$ (95% CI, 0.907–0.965; $p < 0.0001$) and $r = 0.927$ (95% CI, 0.900–0.947; $p < 0.0001$), respectively.

In **Figure 3**, a scatter plot is constructed with System 2's results along the x-axis and System 1's along the y-axis to show the distribution of the sIgE concentration results from the two systems for *D. pteronyssinus*, *D. farinae*, cat dander, milk, shrimp, and peanut. For a concordance and discordance analysis, the allergens that showed ± 1 class difference were *D. pteronyssinus* (91.60%, 229 vs. 250), *D. farinae* (81.25%, 91 vs. 112), cat dander (98.00%, 147 vs. 150), milk (83.58%, 112 vs. 134), shrimp (59.72%, 129 vs. 216), and peanut (76.56%, 49 vs. 64). The combined total concordance rate was 81.75% (757 vs. 926).

FIGURE LEGENDS:

Figure 1: The positivity rates of the detection of 19 common allergens by the microfluidic assay. d1 = *Dermatophagoides pteronyssinus*, d2 = *Dermatophagoides farinae*, d201 = *Blomia tropicalis*, e1 = cat dander, e5 = dog dander, g2 = Bermuda grass, g6 = timothy grass, i6 =

cockroaches, m3 = *Aspergillus fumigatus*, m5 = *Candida albicans*, w1 = ragweed, f1 = egg white, f2 = milk, f4 = wheat, f13 = peanut, f14 = soybean, f20 = almond, f23 = crab, and f24 = shrimp.

Figure 2: Levey-Jennings graphs of the three allergens repeatedly detected by the microfluidic system. (A) Cat hair, (B) dog hair, and (C) cockroach were selected for repeatability evaluation. The black, green, yellow, and red lines represent the mean (\bar{X}), the mean \pm the standard deviation ($\bar{X} \pm SD$), the mean + the standard deviation times two ($\bar{X} \pm 2SD$), and the mean + the standard deviation times three ($\bar{X} \pm 3SD$) of multiple measurements, respectively.

Figure 3: Scatter plots of six allergen sIgE concentrations measured by System 1. System 1 (Y-axis) and the System 2 System (X-axis). Each line in the plot represents class cutoffs (class 0: 0.35, class 1: 0.35–0.7, class 2: 0.7–3.5, class 3: 3.5–17.5, class 4: 17.5–50, class 5: 50–100, and class 6: >100 kUA/L). Shaded boxes are concordant areas in the concentration class.

Table 1: Patient demographic characteristics. In total, 293 subjects were found who fulfilled the inclusion criteria, with an average age of 23 (with a range from 8 to 36 years old). Among them, 170 (58.02%) were male and 123 (41.98%) were female. Also, 92 (31.40%) of them had allergic rhinitis, 117 (39.93%) had allergic asthma, 36 (12.29%) had comorbidity of rhinitis and asthma, and 48 (16.38%) had other allergic diseases, such as a food allergy or skin allergies.

Table 2: Clinical performance between the two systems. d1 = *D. pteronyssinus*, d2 = *D. farina*, e1 = cat dander, f2 = milk, f13 = peanut, and f24 = shrimp. CAP = ImmunoCAP, + = positive, – = negative, SE = sensitivity, SP = specificity, PPV = positive predictive value, NPV = negative predictive value, AUC = area under the ROC curve. For the AUC values, the 95% interval value (95%, CI) is also shown in the table.

Table 3: Correlation and agreement between the two systems. d1 = *D. pteronyssinus*, d2 = *D. farina*, e1 = cat dander, f2 = milk, f13 = peanut, and f24 = shrimp. For kappa and Spearman's Rho values, the 95% interval value (95%, CI) is also shown in the table.

DISCUSSION:

Similar to the results from many other studies^{15–17}, the results from the microfluidic system based on sera from 293 allergic patients showed that house dust mites (including *D. pteronyssinus*, *D. farinae*, and *B. tropicalis*) are the main inhalant allergens leading to allergic diseases in southern China, whereas for food, milk, peanut, shrimp, and crab are the commonest allergens that cause allergic symptoms. With regard to the reproducibility study done on three allergens, all of them showed good results, with an overall repetition rate of 88.89%, which means it met the maximum allowable error.

Using System 1 as reference, the current study evaluated the clinical diagnosis efficacy of the System 2. With a serum sIgE level of 0.35 IU/mL as cutoff², a sample with sIgE > 0.35 IU/mL implies that the patient is sensitive to the allergen, and the higher the titer, the better correlation to the patient's symptoms¹⁸. The results show that the concordance rate of the

three inhalant allergens were all more than 90%. In addition to the results for food allergens, which had a concordance of ~40.74%–72.39%, the total concordance was 81.75% (757/926). The kappa value of *D. pteronyssinus*, *D. farina*, and cat dander were 0.778, 0.663, and 0.860 ($p < 0.001$), respectively. The kappa values for food allergens were all below 0.4. For the three major inhalant and food allergens, a significant correlation of quantitative results was seen between the two systems ($r_{\text{Spearman}} \approx 0.681\text{--}0.969$, $p < 0.01$).

It was noticed that while the r_s coefficient for peanut was 0.969, the kappa value of the consistency evaluation index was only 0.349 (95% CI, 0.129–0.569). Such discrepancy might be due to the low prevalence of peanut sensitivity in the region and, hence, most of the recruited sera were negative for that specific IgE. Many studies have indicated that a significant discrepancy could be seen between sIgE titer and clinical symptoms of food allergens. The use of different assay systems for food-specific IgE determination could also create big variations¹⁹. This might be due to the fact it is not the raw ingested food which triggers the allergic symptoms, but the modified components generated during cooking or digestion. The use of different raw materials by different manufacturers to make the allergens can also contribute to the result discrepancy²⁰.

The microfluidic cartridge is composed of five major parts: five storage tanks, five reagent delivery channels, five unidirectional pumps, a single reaction zone in which allergen extracts can be immobilized, and a waste tank to collect all reaction by-products. Based on the assay need, up to 40 allergen extracts can be dotted on the reaction zone. Controlled by the PC, the five unidirectional pumps guide and coordinate the flow of serum sample, washing buffer, blocking reagent, conjugates, and subtracts, to finish a two-step enzyme-linked immunosorbent assay. After the reaction is completed, the chemiluminescence reaction images are captured by a low-resolution cooled CCD camera and the signals are processed by the PC to establish the calibration curve and to calculate quantitative and semiquantitative sIgE results.

The current study shows that the two systems demonstrate good consistency. However, compared with the System 1, the System 2 is easier to use and has a lower demand for operator training. Since each microfluidic cartridge has its own quality control curve, the reliability of the system is greatly enhanced. Other advantages of the system include a light and small footprint, an expandable modular setup, and the ease with which it is connected to a PC for operation control. All these advantages greatly reduce the setup and running cost, and at the same time, they do not jeopardize the accuracy and speed requirements in daily clinical practice, which makes the system particularly suitable for allergy screening in primary care hospitals in China. Nevertheless, one major drawback of the microfluidic system is that it is not a fully automatic system, and frequent intervention is needed during the operation. It still cannot replace the systems that need to process a big number of samples daily.

Due to the lack of enough positive sera for certain allergens, this study did not cover all the 19 allergens available in the microfluidic cartridge, but just six common ones available in southern China. More study is needed to elaborate on whether the evaluation is also

applicable to other allergens.

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DISCLOSURES:

The authors have nothing to disclose.

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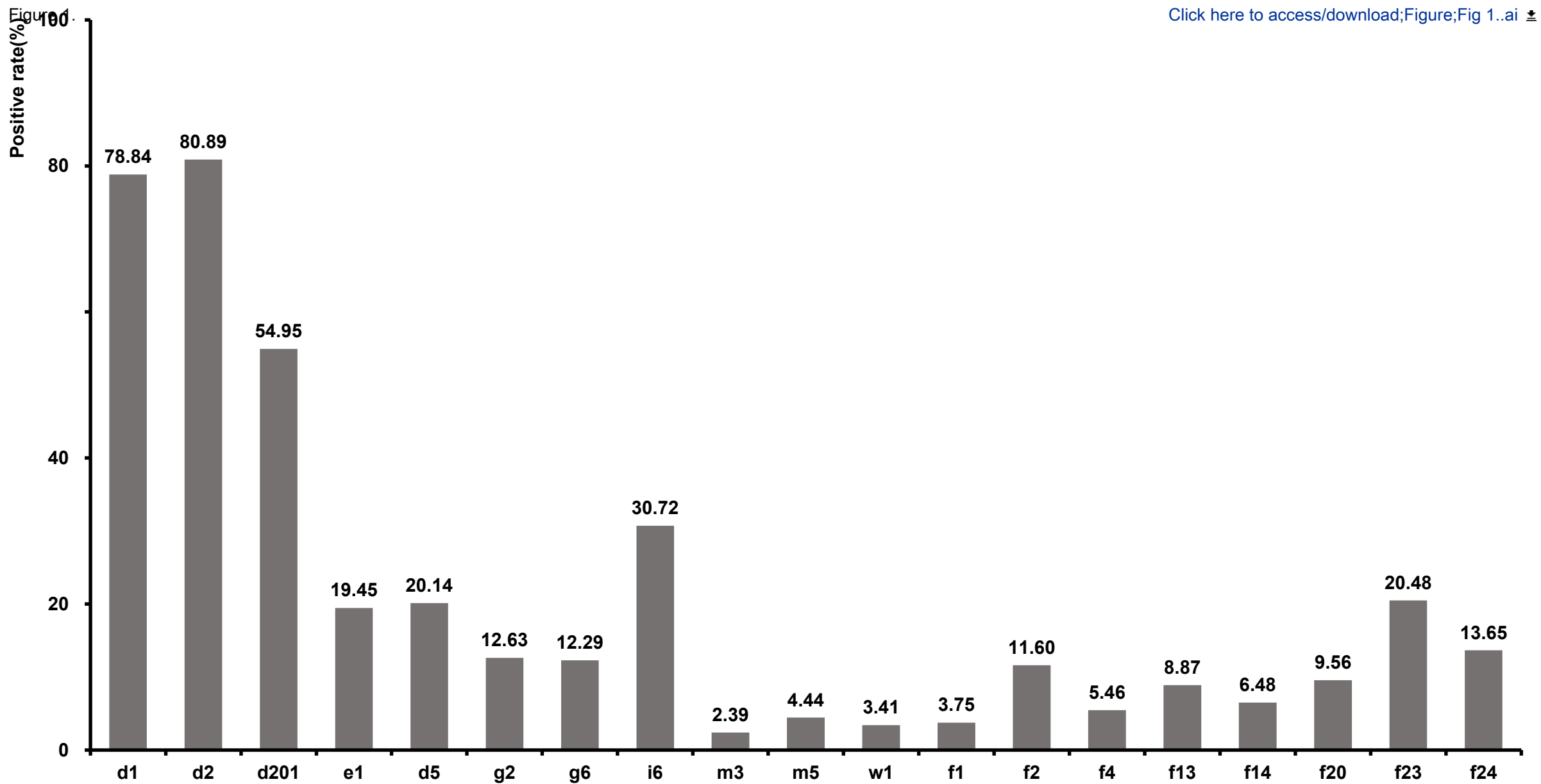
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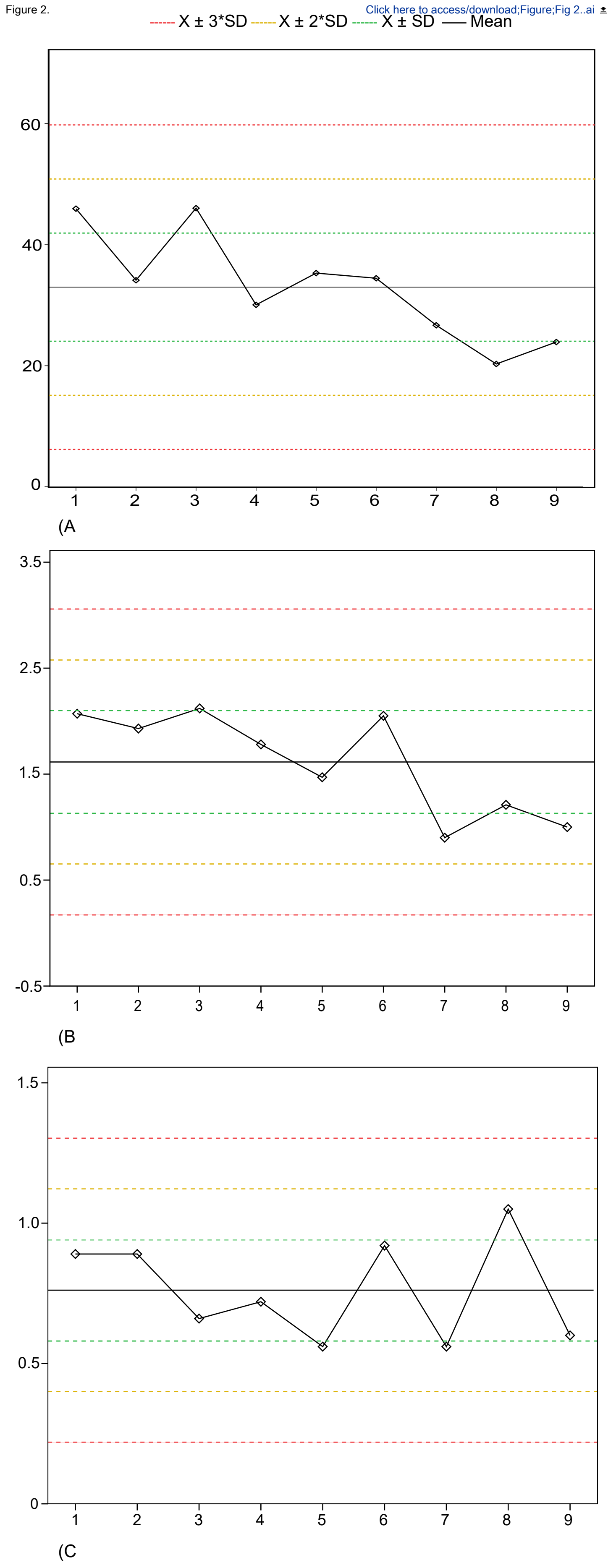
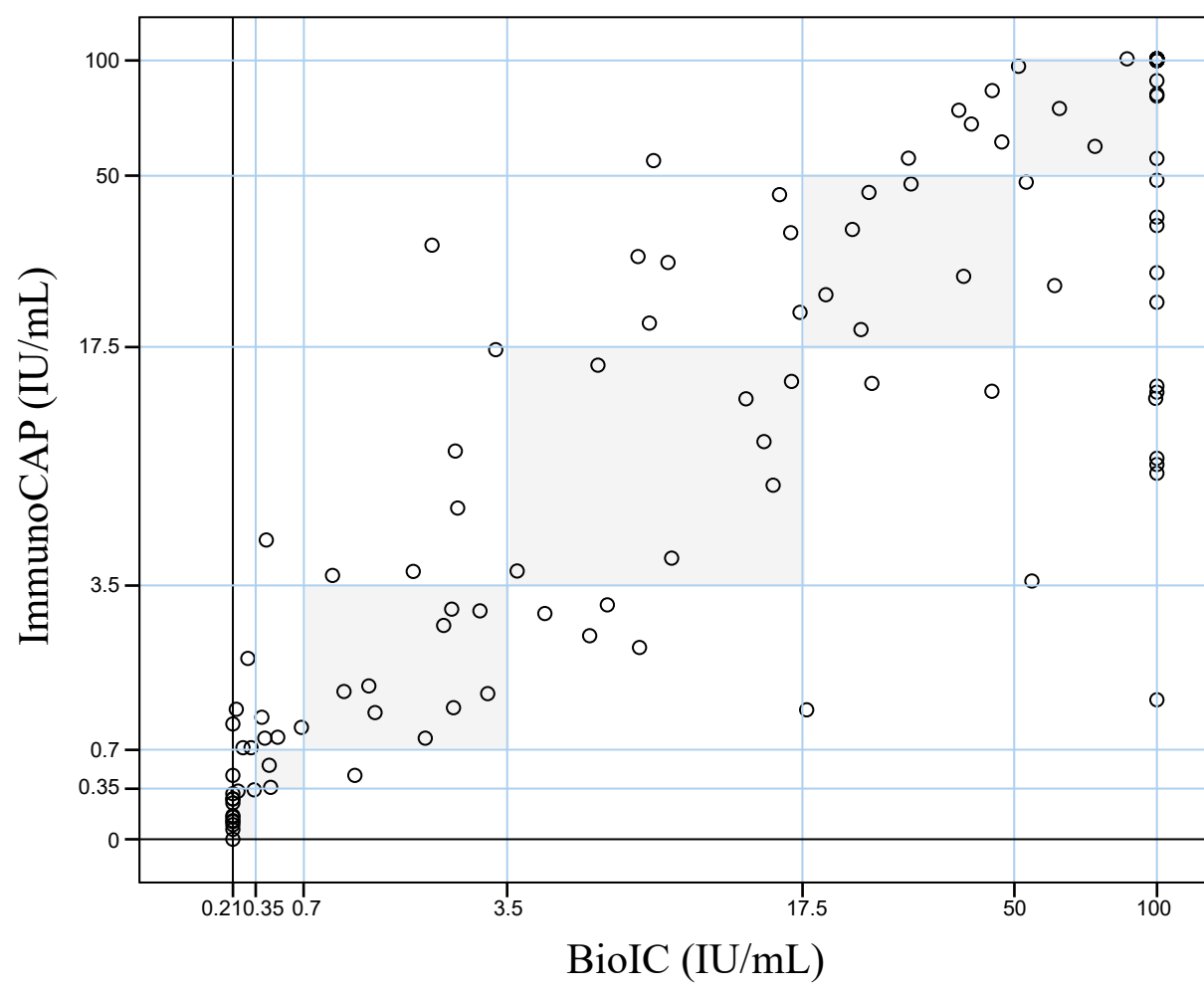


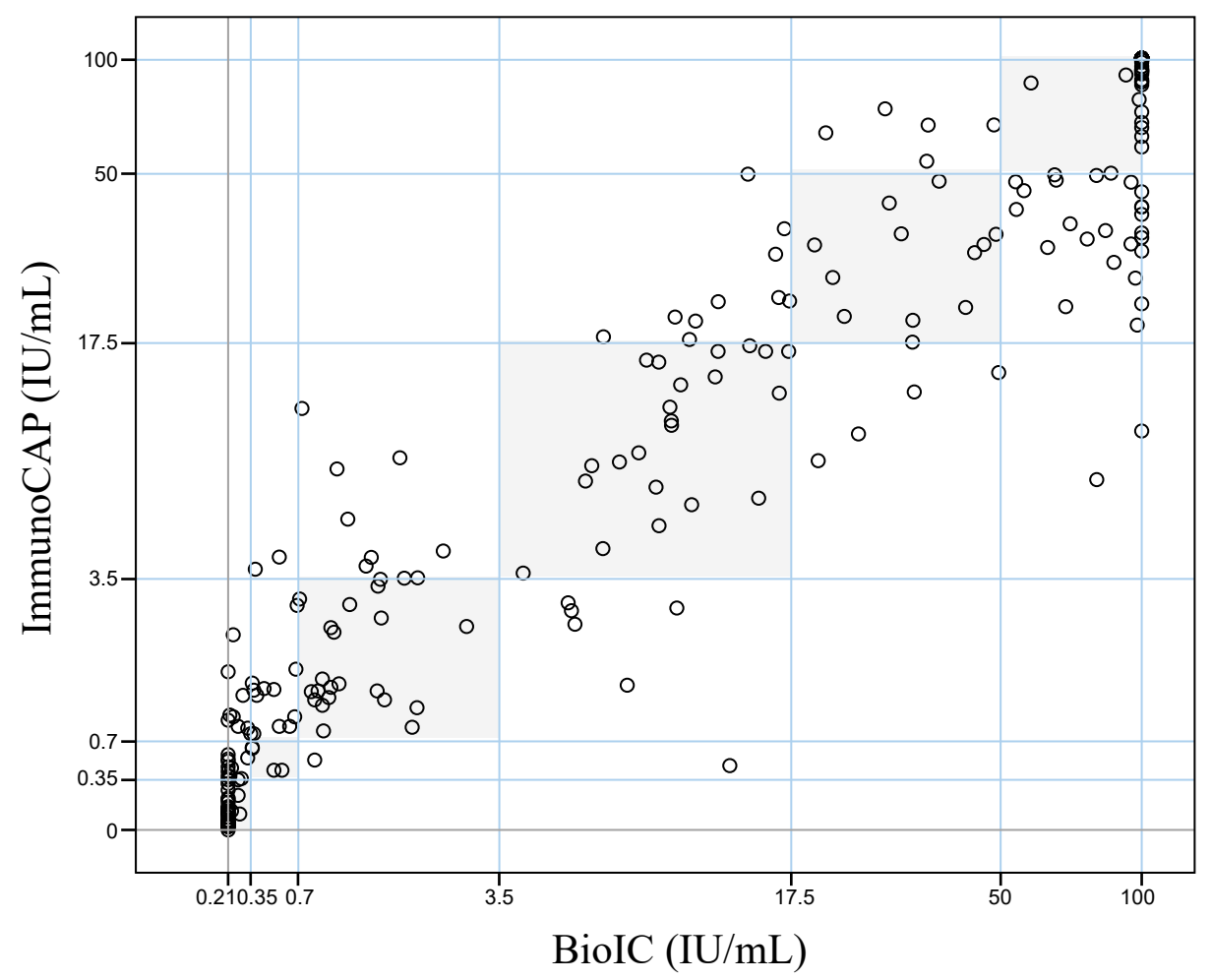
Figure 3.

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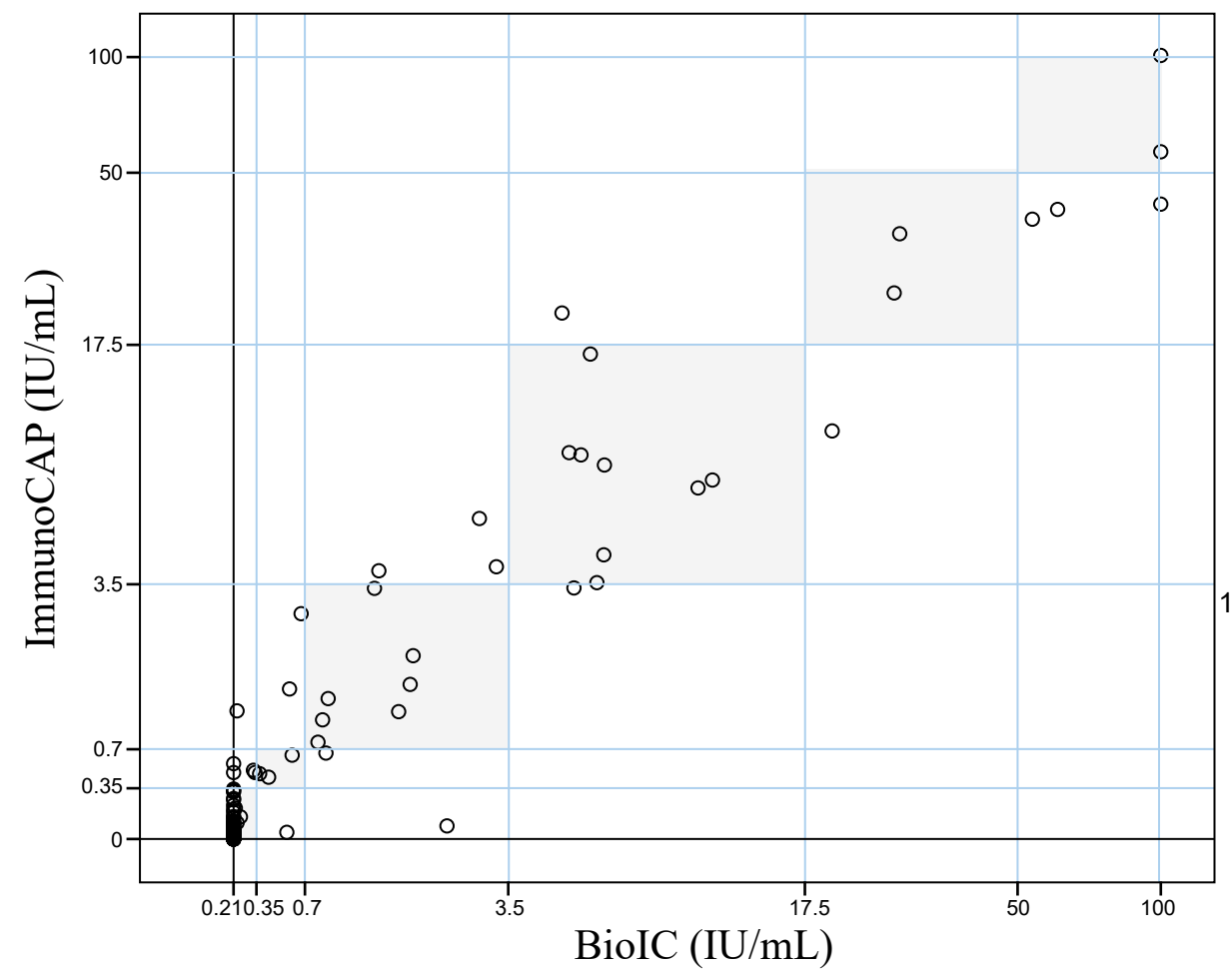
Dermatophagoides pteronyssinus



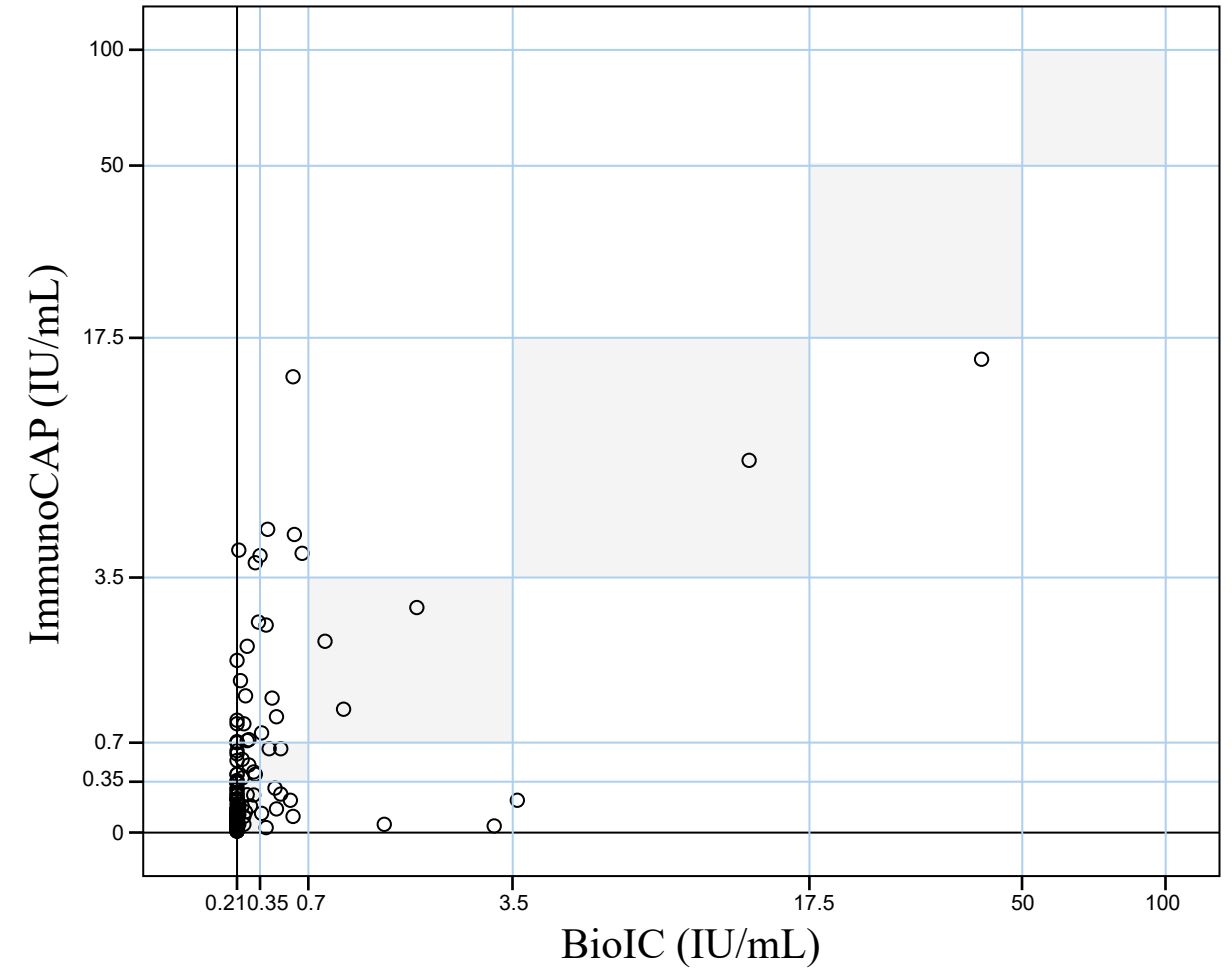
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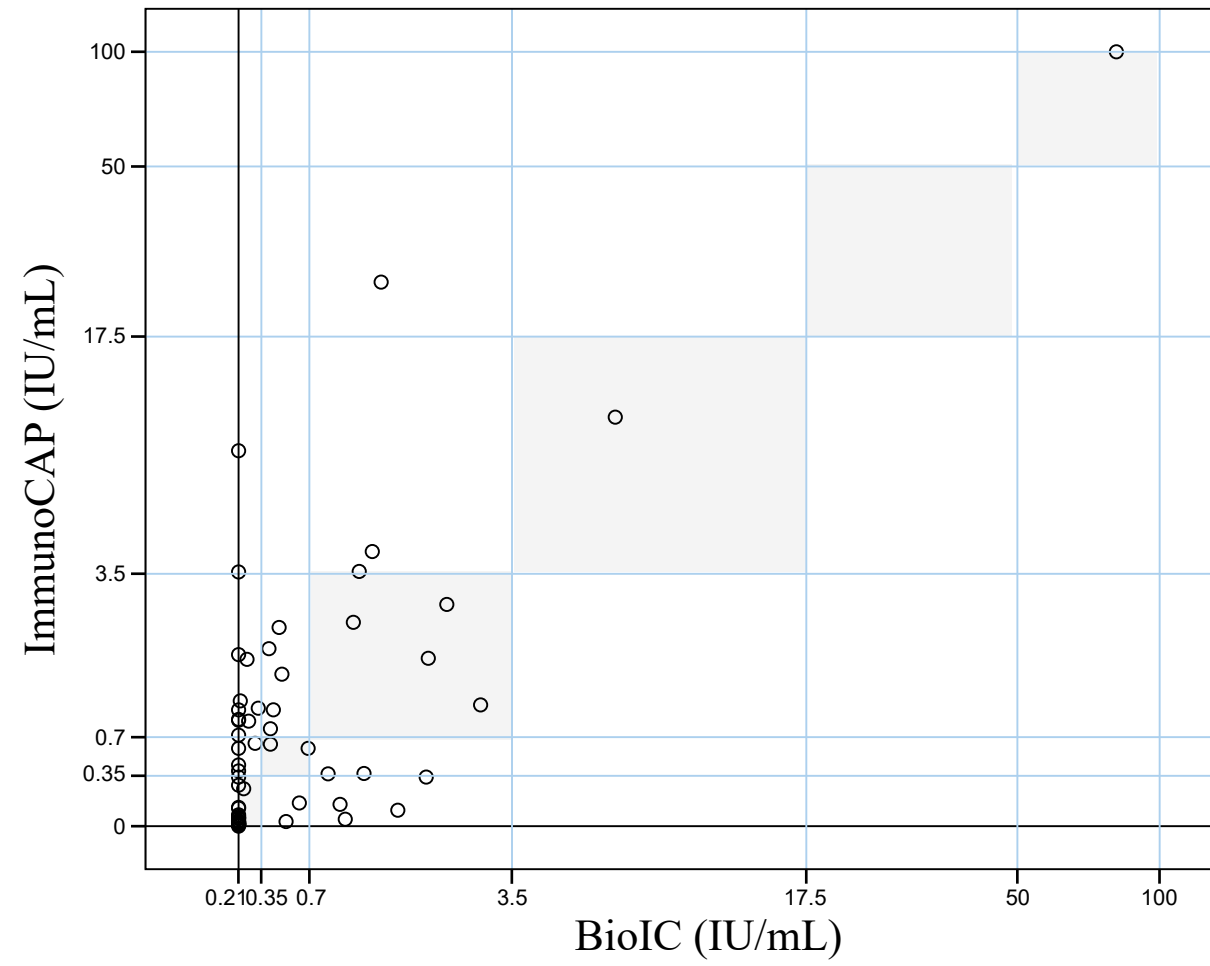
Cat dander



Milk



Peanut



Shrimp

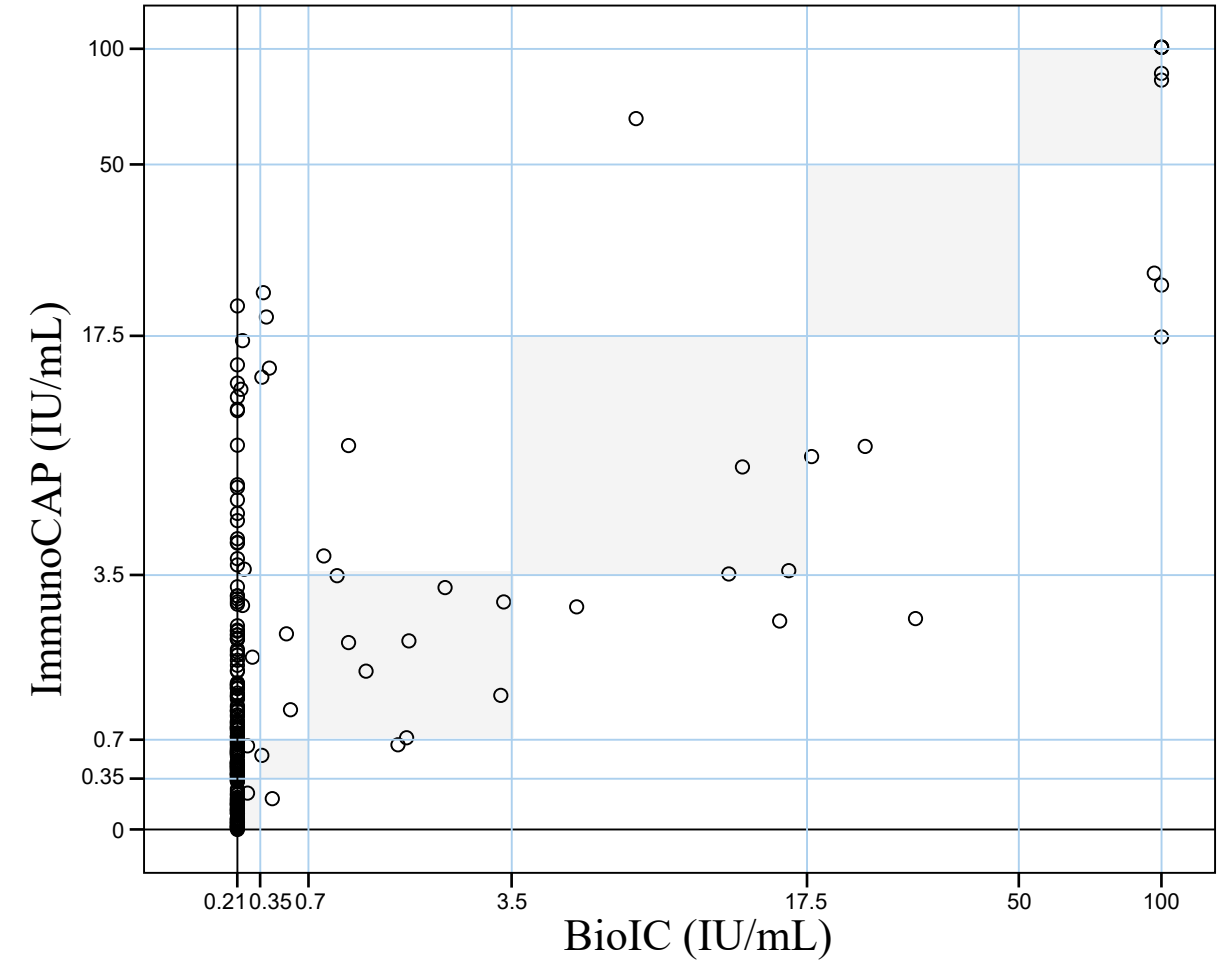


Table 1. Patient Demographic Characteristics.

Characteristic	No.(%)
Gender, n(%)	
Female	123(41.98%)
male	170(58.02%)
Age, year, n(%)	
Median (25%,75%)	23(8,36)
≤10	97(33.11%)
11-20	37(12.63%)
21-40	101(34.47%)
>41	58(19.80%)
Diagnosis, n(%)	
Allergic rhinitis	92(31.40%)
Allergic asthma	117(39.93%)
Allergic rhinitis with asthma	36(12.29%)
Others	48(16.38%)

Totally 293 subjects were found to fulfill the inclusion criteria, with an average age of 23 (range from 8 to 36 years old). Among them, 170(58.02%) were male and 123(41.98%) were female. 92(31.40%) of them had allergic rhinitis, 117(39.93%) had allergic asthma, 36(12.29%) with comorbidity of rhinitis and asthma, and 48(16.38%) had other allergic diseases such as food allergy, skin allergies, etc.

Table 2. Clinical performance between the two systems.

	Sample size	CAP +		CAP -		Total agreement	SE	SP	PPV	NPV	AUC(95%,CI)
		BioIC +	BioIC -	BioIC +	BioIC -						
d1	250	196	20	0	34	92.00%	90.74%	100.00%	100.00%	62.96%	0.975(0.947 to 0.991)
d2	112	93	6	0	13	94.64%	93.94%	100.00%	100.00%	68.42%	0.984(0.941 to 0.999)
e1	150	34	5	2	109	95.33%	87.18%	98.20%	94.44%	95.61%	0.968(0.925 to 0.990)
f2	134	16	27	10	81	72.39%	37.21%	89.01%	61.54%	75.00%	0.744(0.661 to 0.815)
f13	64	18	15	6	25	67.19%	54.55%	80.65%	75.00%	62.50%	0.731(0.606 to 0.834)
f24	216	36	127	1	52	40.74%	22.09%	98.11%	97.30%	29.05%	0.613(0.545 to 0.678)

d1-Der. p1, d2- Der. f1, e1-Cat dander, f2-Milk, f13-Peanut, f24-Shrimp. CAP-ImmunoCAP, +-positive, --negative, SE-sensitivity, SP-specificity, PPV-positive predictive value, NPV-negative predictive value, AUC-area under the ROC curve. For AUC values, the 95% interval value (95%, CI) is also shown in the table.

Table 3. Correlation and agreement between the two systems.

	<i>Kappa(95%,CI)</i>	<i>Spearman`rho(95%,CI)</i>
<i>d1</i>	0.727(0.617 to 0.838)	0.896(0.869 to 0.918)
<i>d2</i>	0.783(0.617 to 0.948)	0.731(0.631 to 0.807)
<i>e1</i>	0.876(0.786 to 0.965)	0.927(0.900 to 0.947)
<i>f2</i>	0.293(0.122 to 0.463)	0.681(0.579 to 0.763)
<i>f13</i>	0.349(0.129 to 0.569)	0.969(0.949 to 0.981)
<i>f24</i>	0.112(0.062 to 0.162)	0.833(0.788 to 0.870)

d1-Der. p1, d2- Der. f1, e1-Cat dander, f2-Milk, f13-Peanut, f24-Shrimp. For kappa and Spearman` rho values, the 95% interval value (95%, CI) is also shown in the table.

Name of Material/ Equipment	Company	Catalog Number	Comments/Description
Agnitio BioIC Analyzer	Agnitio Science & Technology(Taiwan, China)	BA-G2000	
BioIC Allergen specific-IgE Detection Kit	Agnitio Science & Technology(Taiwan, China)	DR17A12	
BioIC Cartridge Placement plate	Agnitio Science & Technology(Taiwan, China)	T20SET	
BioIC Reagent Dispenser	Agnitio Science & Technology(Taiwan, China)	DS-1	
Image two-dimensional barcode machine	Agnitio Science & Technology(Taiwan, China)	NLS-HR200	
Software Package, LabIT	Agnitio Science & Technology(Taiwan, China)	Version 2.4.12	
VORTEX-5 Vortex Mixer	Haimen Kylinbell Lab Lastruments Co., Ltd.	VORTEX-5	



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CORRESPONDING AUTHOR

Name:

Prof. Baoqing Sun

Department:

Allergy and Clinical Immunology

Institution:

The First Affiliated Hospital of Guangzhou Medical University, Guangzhou Medical University, Guangzhou, China.

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[Answer 1: We have re-corrected the words and grammar of the full text to ensure that no such problems have occurred.](#)

2. In editorial manager, you selected standard access. However, in the author license agreement, the open access box is checked. Please use a new form, check the standard access box, sign it and upload it to your editorial manager account when you submit your revision.

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[Answer 3: Revised as required. After repeated consideration, we have minimized the use of the term "BioIC" in the full text, or use other words instead. But we think that "BioIC"](#)

represents a method/system without commercial meaning, just like "ImmunoCAP".

4. Please ensure that all text in the protocol section is written in the imperative tense as if telling someone how to do the technique (e.g., "Do this," "Ensure that," etc.).

5. Step 1.1: Please write this step in the imperative tense.

6. 1.2: Please write this step in the imperative tense.

7. 1.3.1-1.3.6: Please write each step in complete sentences and in the imperative tense.

8. 1.5: Please write this step in the imperative tense.

9. 2.1: Please write this step in the imperative tense.

10. 2.2: Please write this step in the imperative tense.

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12. 4.1: Please write this step in the imperative tense.

13. 4.2: Please write this step in the imperative tense.

14. 5.1: Please write this step in the imperative tense.

15. 5.2: Please write this step in the imperative tense.

16. 5.3: Please write this step in the imperative tense.

17. 5.4: Please write this step in the imperative tense.

Answer 4-17: we have all revised as the above requires. Please see the Protocol section/line 71-201.

PROTOCOL

This study and the use of the human serum samples have obtained approval from the Ethics Committee of The First Affiliated Hospital of Guangzhou Medical University (GYYY-2016-73). All participants have given their written consent independently or through their parents (in the case of children).

1. Basic information of the study group

NOTE: The Allergy Information Repository of the State Key Laboratory of Respiratory

Disease (AIR-SKLRD) is a large serum bank established inside the Guangzhou Institute of Respiratory Hospital (GIRH). Started from the last decade, AIR-SKLRD has already started to collect and store serum samples from patients with allergic diseases, together with their clinical information^{4,5}. The current study was performed with the sera from AIR-SKLRD.

1.1. Search the database for sera collected from January 2015 to June 2018 in AIR-SKLRD and select the patients with allergic disease and were found to be sensitive to the common allergens in the region.

1.2. Ensure that all selected patients have allergic-related diseases such as allergic rhinitis and or asthma, allergic dermatitis, urticaria, And the serum of these patients were multiple sIgE sensitization of common allergen in this region detected by ImmunoCAP method.

1.3. Exclude patients with incomplete medical records, those lost to follow up, those who refuse to give informed consent regarding the use of their serum samples for scientific purposes, those with identified immunodeficiency, those currently on immunotherapy or immunomodulatory agents, or those found to have parasitic infections.

1.4. Ensure no treatment or drug prescription was given prior to serum collection so as to minimize the interference to the laboratory findings. All serum samples not fulfilling the criteria were rejected.

2. Study flow and measurements of interest

NOTE: Use the microfluidic System needs 100 µl serum for determining 19 allergens. Venous blood (5 mL) was collected from each patient using a vacuum blood vessel containing separating gel. After centrifuging for 10 minutes at 1000 (x g), the upper layer was collected for testing. Unused serum was stored at -80°C. Prior to testing, serum was kept at room temperature for 30 minutes and was shake with vortex mixer. Repeated freeze and thaw were avoided.

2.1. Primarily test the serum samples for sIgEs to whole allergens of Dermatophagoides pteronyssinus (d1), Dermatophagoides farinae (d2), Blomia tropicalis (d201), cat dander (e1), dog dander (e5), Bermuda grass (g2), timothy grass (g6), cockroaches (i6), Aspergillus fumigatus (m3), Candida albicans (m5), ragweed (w1), egg white (f1), milk (f2), wheat (f4), peanut (f13), soybean (f14), almond (f20), crab (f23) and shrimp (f24).. Follow the instructions in Section 3. Note: sIgE determination was done with the allergen specific IgE assay kit (see table of materials) and measured by a chemiluminescence analyzer.

2.2. Randomly select three samples among the samples with enough serum (at least

900 µl) for reproducibility study. Keeping all the conditions unchanged, measured the three sera for allergen sIgEs daily for 9 consecutive days, i.e. totally $100 \times 9 = 900$ µl serum.

3. Semi- automation test procedure of the microfluidic System

NOTE: BioIC system is the integration of automatic microfluidic technology, protein microarray, cold light analysis, parallel IgE analysis and image processing technology. The testing protocol is divided into four parts: preparation of equipment, sample loading, incubation, and measurement.

3.1. Preparation of equipment

3.1.1. Turn on the PC and analyzer power. Note: The power switch is on the left of the base.

3.1.2. Start the LabIT program on the PC. If the “Dark Frame” warning window pops up, click “OK” to run LEAK Test. Afterwards, click the center logo to enter the operation interface.

Note: the system will remind user to run LEAK Test if it is idle for more than 24 hours.

3.1.3. Check the Reaction Temp and CCD (Charge-coupled Device) Temp at the lower right corner of the screen. The Reaction Temp should rise to $37\text{ }^{\circ}\text{C} \pm 1\text{ }^{\circ}\text{C}$ in about 10 minutes, and the CCD Temp should drop to $-15\text{ }^{\circ}\text{C} \pm 1\text{ }^{\circ}\text{C}$.

3.1.4. Run the LEAK Test after the CCD Temp has dropped to $-15\text{ }^{\circ}\text{C} \pm 1\text{ }^{\circ}\text{C}$. Before running the LEAK test, make sure there is no other items left inside the instrument and close the door. Click Tools > System Test > LEAK Test. Do not open the door during the testing. When the test is finished, the report window will pop up.

3.2. Sample loading

3.2.1. Add 620 µl wash buffer, 120 µl blocking buffer, 60 µl of conjugates A and B, 60 µl of substrate A and B, and 100 µl of serum samples to the corresponding reagent tank on the microfluidic cartridge.

3.3. Incubation

3.3.1. Click on “Cartridge ID”, use the barcode scanner to scan the serial number of the cartridge, enter sample ID, put the cartridge into the analyzer and close the door, click “Analyzer” and “Run” to start the analysis.

3.4. Measurement

3.4.1. Export the result to statistical software (e.g., Excel) after measurement. Note: after 30 minutes of incubation, the analyzer automatically performs the measurement and reports the result.

3.5. Switch off the analyzer

3.5.1. Routine maintenance: After finishing the test, remove the cartridge and wipe the analyzer's internal heating iron and electromagnet lightly with 75% alcohol. Note: Do not press hard or shake the electromagnet.

3.5.2. Close the LabIT window. The temperature monitoring window will pop up. It will automatically close when the CCD warms up to the 5 °C protection mode. By then, it will be safe to turn off the power of the analyzer and PC. Note: Don not manually close the temperature monitoring window before the CCD Temp has risen to 5 °C, and don't turn off the power of the analyzer nor the PC during the CCD warm up.

4. Definition of sIgE reactivity

NOTE: For an undiluted serum specimen, the detection range of BioIC System is 0.21-100 IU/ml.

4.1. Based on the threshold value of 0.35 IU/ml, consider an sIgE level exceeding 0.35 IU/ml to be positive^{6,7}. Rate the reactivity of the sIgE tests as⁸: class 1 (≥ 0.35 and < 0.70 IU/ml), class 2 (≥ 0.70 and < 3.50 IU/ml), class 3 (≥ 3.50 and < 17.50 IU/ml), class 4 (≥ 17.50 and < 50.00 IU/ml), class 5 (≥ 50.00 and < 100.00 IU/ml), and class 6 (≥ 100.00 IU/ml).

5. Statistical analysis

5.1. Use a histogram to show the positive rate of the 19 allergens and use the Levey-Jennings curve to demonstrate the repeatability of the detection system⁹.

5.2. Select three commonest Inhalant allergens and food allergens (totally six allergens) and compare to the ImmunoCAP to evaluate its clinical diagnostic performance^{10,11}. Use include concordance rate, sensitivity, specificity, positive and negative predictive values and the area under the receiver operating characteristic (ROC) curve (AUC) as the evaluation criteria.

5.3. Apply the spearman's correlation analysis¹² to describe correlations between the two systems. And Kappa value for Consistency. Categorize the kappa value as almost perfect (0.8-1.0), substantial (0.6-0.8), moderate (0.4-0.6), fair (0.2-0.4) or poor (< 0.2)¹³. Use SPSS 23.0 and MedCalc 11.0 statistical analysis and define $P < 0.05$ as statistical significance.

18. Figure 2: Please add a short description of the figure in Figure Legend.

Answer 18: Revised as required. please see line 256-261.

Figure 2. Levey-Jennings graphs of the 3 allergens repeatedly detected by the microfluidic System.

Cat hair (A), dog hair (B), and cockroach (C) were selected for repeatability evaluation. The black, green, yellow and red line represent the mean (X), mean \pm standard deviation (X \pm SD), mean + twice of standard deviation (X \pm 2SD), mean + three times of standard deviation (X \pm 3SD) of multiple measurements, respectively.

19. Each table must be accompanied by a title and a description after the Representative

Results of the manuscript text.

Answer 19: Revised as required. please see line 269-284.

Table 1. Patient Demographic Characteristics.

Totally 293 subjects were found to fulfill the inclusion criteria, with an average age of 23 (range from 8 to 36 years old). Among them, 170(58.02%) were male and 123(41.98%) were female. 92(31.40%) of them had allergic rhinitis, 117(39.93%) had allergic asthma, 36(12.29%) with comorbidity of rhinitis and asthma, and 48(16.38%) had other allergic diseases such as food allergy, skin allergies, etc.

Table 2. Clinical performance between BioIC and ImmunoCAP assays.

d1-Der. p1, d2- Der. f1, e1-Cat dander, f2-Milk, f13-Peanut, f24-Shrimp. CAP-ImmunoCAP, +-positive, —negative, SE-sensitivity, SP-specificity, PPV-positive predictive value, NPV-negative predictive value, AUC-area under the ROC curve. For AUC values, the 95% interval value (95%, CI) is also shown in the table.

Table 3. Correlation and agreement between BioIC and ImmunoCAP assays.

d1-Der. p1, d2- Der. f1, e1-Cat dander, f2-Milk, f13-Peanut, f24-Shrimp. For kappa and Spearman` rho values, the 95% interval value (95%, CI) is also shown in the table.

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FirstPage – LastPage, (YEAR).

Answer 20, 21: Revised as required. The format of all references has been modified, please see the Reference section.

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For more improvements, please see the revised manuscript. Thank you for giving me the opportunity to modify and submit again.

Best sincerely,

Zhifeng Huang M.D

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