

A Simple and Rapid Light-Initiated Chemiluminescence Assay for Quantitation of *Artemisia*-Specific Immunoglobulin E

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Keywords

Artemisia pollen allergy · Specific immunoglobulin E · Light-initiated chemiluminescence assay · Quantitative detection · Homogeneous immunoassay

Abstract

Background: Light-initiated chemiluminescence assay (LICA) is a homogeneous assay that has been successfully used for the quantitation of food allergen-specific immunoglobulin E (sIgE), but not inhaled allergen-sIgE. Simultaneously, current assays used to detect allergen-sIgE are serum consuming and/or time consuming. Hence, we established a method for the quantitation of *Artemisia*-sIgE based on LICA and verified its performance according to the clinical guideline documents, laying a foundation for the quantitation of inhaled and food allergen-sIgE in parallel on LICA.

Methods: The assay was established after optimizing the first incubation time and the dilutions of *Artemisia*-coated chemibeads, biotinylated goat anti-human IgE, and serum. In order to quantitate *Artemisia*-sIgE, the calibration curve was established with a high positive serum of known concentration. The assay performance was confirmed per the clinical guideline documents. In addition, the correlation between the results of LICA and capture enzyme-linked immu-

nosorbent assay was evaluated. **Results:** The developed LICA's coefficients of variation of repeatability and intermediate precision were 3.20%, 2.14%, and 3.85% and 4.30%, 4.00%, and 4.40%, respectively. The limit of detection was 0.10 kU_A/L, and the limit of quantitation was 0.11 kU_A/L. The range of linearity was from 0.27 kU_A/L to 97.53 kU_A/L ($r = 0.9968$). The correlation coefficient (r) for the correlation analysis between results of LICA and capture ELISA was 0.9087. This assay was successfully applied in 64 human serum samples, showing good sensitivity (82.20%) and specificity (100%). **Conclusion:** An *Artemisia*-sIgE quantitation assay based on LICA was successfully established. Its performance satisfied the clinical requirements and could be widely used in clinical laboratories.

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Introduction

“Wash-free” immunoassay platforms, which depend on the ordinary “mix-and-measure” protocol, are especially attractive due to rapid reaction, simplification, and

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automation adaptability. In the past few years, no-wash immunoassay platforms have been increasingly established, including colorimetric assay based on a target-induced aggregation of Au or Ag nanoparticles, fluorescence polarization immunoassay, fluorescence resonance energy transfer immunoassay, homogeneous chemiluminescent immunoassay, and luminescence oxygen channeling immunoassay (LOCI). Among these methods, LOCI, known as AlphaLISA and AlphaScreen, has been widely used in singleplex analyte immunoassays [1]. Similar to LOCI, light-initiated chemiluminescence assay (LICA) is a bead-based immunoassay. The immunoassay contains 2 kinds of beads: chemibeads and sensibeads. Sensibeads generate a flow of singlet oxygen at the excitation of 680 nm, which can travel approximately 200 nm in a liquid phase environment. When chemibeads and sensibeads approach each other (<200 nm distance), the singlet oxygen could be absorbed by chemibeads and then produce a strong fluorescent signal at 520–620 nm [2–5]. In addition, the LICA platform can provide an efficient way for quantitative detection without complex reagent loading and washing steps as in conventional immunoassays.

Nowadays, LICA has been used in many studies, such as detection of hepatitis C virus antibodies [6], total testosterone [7], estradiol [8], human cytomegalovirus immunoglobulin G (IgG) antibodies [9], allergen-specific IgG4 antibodies [10], and *Treponema pallidum* antibodies [11]. Apart from the above, our team had successfully applied LICA to the detection of food allergen-specific immunoglobulin E (sIgE), such as egg-sIgE [12] and cow's milk-sIgE [13]. Previously, we established an LICA-based method for component-resolved diagnostic of egg allergy. The egg-sIgE-LICA assay possessed a range of important performance properties such as high sensitivity and reproducibility, small sample volume, short assay time, simple operation, larger positive to negative ratio, and liquid phase reaction environment [12]. We also developed an LICA-based assay for the detection of cow's milk-sIgE. Furthermore, we proved that the ability of LICA to resist cow's milk-specific IgG interference is superior to the indirect ELISA method in the detection of cow's milk-sIgE [13].

To the best of our knowledge, there has been no report on the LICA-based detection of inhaled allergen-sIgE till date [6–13]. Moreover, the *Artemisia* pollen is the most widespread outdoor allergen throughout China, which can cause allergic rhinitis, asthma, and/or both [14]. Therefore, we took *Artemisia* as an example to establish an LICA-based quantitative detection assay for inhaled

allergen-sIgE that can replace ImmunoCAP in an affordable way, laying a practical foundation for LICA-based quantitation of inhaled and food allergen-sIgE in parallel. Besides, we will verify the performance of this assay per the clinical guideline documents to confirm that it can be used in clinical laboratories.

Materials and Methods

Samples

Serum samples, collected from the Second Affiliated Hospital of Tianjin University of Traditional Chinese Medicine and Tianjin Children's Hospital, were stored at -20°C after being filled into coped tubes. *Artemisia*-sIgE of serum samples was tested using capture enzyme-linked immunosorbent assay (ELISA) (HOB, China). Serum samples in which the *Artemisia*-sIgE value was $\geq 0.35 \text{ kU}_A/\text{L}$ were considered positive. The total serum IgE was $>100.00 \text{ kU/L}$ in these samples. On the contrary, serum samples in which the *Artemisia*-sIgE value was $<0.35 \text{ kU}_A/\text{L}$ were considered negative, and the total IgE in these samples was $<100.00 \text{ kU/L}$. Informed consent was obtained from each patient. The study was approved by the Ethics Committee of Tianjin Medical University (TMUHMEC2017008) and conducted according to the principles of the Declaration of Helsinki.

Reagents and Chemicals

Bare chemibeads and streptavidin-coated sensibeads were presented by Beyond Biotech (Shanghai, China). The biotinylated goat anti-human IgE was purchased from Thermo Fisher Scientific (Shanghai, China). The allergen of the *Artemisia* pollen was presented by HOB Biotech (Jiangsu, China). Bovine serum albumin (BSA) was purchased from Solarbio (Beijing, China).

Coupling the *Artemisia* Pollen Allergens with Chemibeads

The *Artemisia* pollen allergens were dissolved in 0.1 M carbonate buffer (CBS) at pH 9.5. Chemibeads were washed with pure water, and the supernatant was discarded after being centrifuged. After the chemibeads were resuspended in CBS, the solution of *Artemisia* pollen allergens was added to it, and the mixture was shaken overnight at 37°C . On the following day, 8 mg/mL NaBH_4 solution was added to the mixture, which was continued to be shaken for 2 h at room temperature (RT) to reduce the beads. Next, the cross-linking was quenched by adding 75 mg/mL glycine, followed by shaking for 1 h at RT. Finally, the *Artemisia*-coated chemibeads were resuspended by the storage solution which contained 25 mM HEPES, 1% BSA, and 0.1% Proclin300, in a final concentration of 10 mg/mL after being washed twice with pure water.

LICA Procedure for Measuring *Artemisia*-sIgE

First, $25 \mu\text{L}$ of the diluted chemibeads, serum, and biotinylated goat anti-human IgE were added to the reaction cups separately and incubated at 37°C . Chemibeads conjugated with *Artemisia* pollen allergen would connect to biotinylated goat anti-human IgE by the effect of serum *Artemisia*-sIgE. Second, $150 \mu\text{L}$ of streptavidin-coated sensibeads were added to each reaction cup and continued to be incubated for 15 min at 37°C . Sensibeads coated with streptavidin would combine with biotinylated goat anti-human

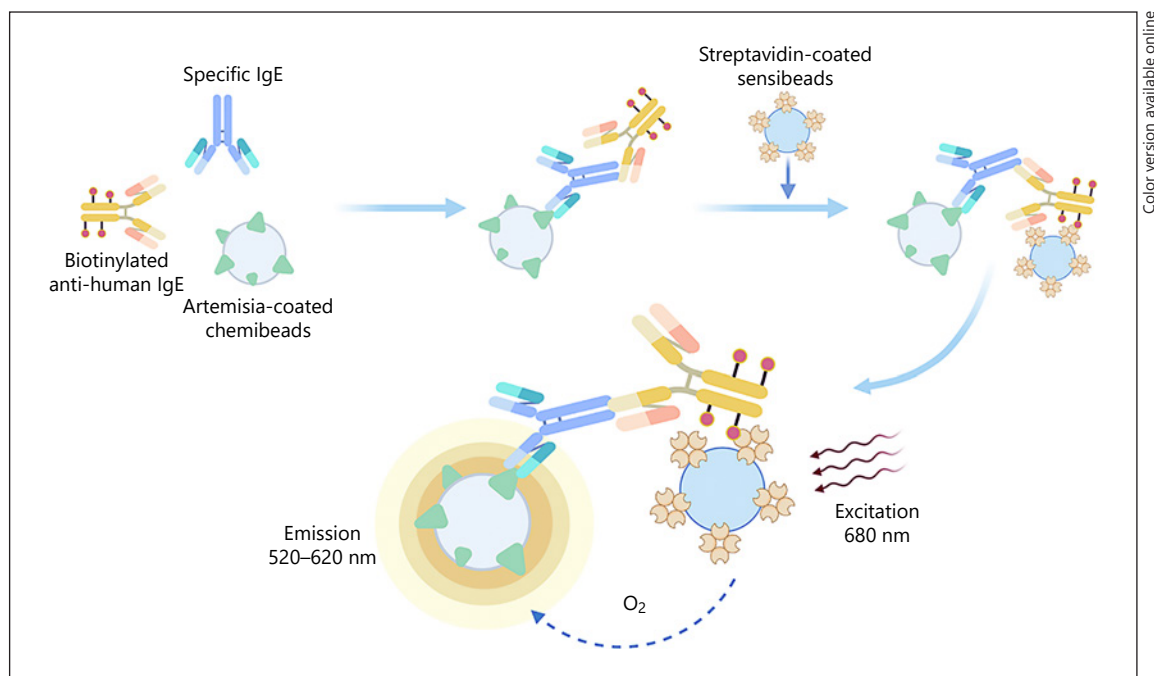


Fig. 1. LICA principle and procedure for measuring *Artemisia*-sIgE. LICA, light-initiated chemiluminescence assay.

IgE. Subsequently, chemibeads were close to sensibeads and able to generate detectable chemiluminescence (CL) signals after being excited (shown in Fig. 1).

Optimization of the Reaction Conditions

The dilution of serum, *Artemisia*-coated chemibeads, biotinylated goat anti-human IgE, and the first incubation time are the main reasons that influence the performance of LICA. First, *Artemisia*-coated chemibeads (10 mg/mL) were diluted to 25 µg/mL, 12.50 µg/mL, and 6.25 µg/mL by 1% BSA. Second, according to the antibodies' instruction, the biotinylated goat anti-human IgE was diluted at the ratio of 1:500, 1:1,000, and 1:2,000 by 1% BSA. Third, the high, medium, and low positive sera were diluted at the ratio of 1:10, 1:20, and 1:40 by 1% BSA. Then, the different incubation times of gradients in the first stage, which are 15 min, 30 min, and 45 min, were studied.

Establishment of the Calibration Curve

A reliable quantitative assay should report the results in units traceable to an international standard. Nowadays, it is infeasible to use an individual-specific IgE for *Artemisia* to perform a homologous calibration method. So, a high positive serum with an *Artemisia*-sIgE concentration of 100 kU_A/L was chosen as the calibrator. Then, the high positive serum was diluted to 17.5 kU_A/L, 3.5 kU_A/L, 0.7 kU_A/L, and 0.35 kU_A/L with negative serum. The concentrations of calibrators were the same as ImmunoCAP.

Precision

Referring to the clinical guideline documents CNAS-GL037 [15] and NCCLS-EP5-A2 [16], 3 positive serum samples with dif-

ferent concentrations of *Artemisia*-sIgE were selected to be tested 20 times in 1 batch by optimized LICA for repeatability. As for intermediate precision, the same above-mentioned samples were tested twice daily for 20 days.

Limit of Detection and Limit of Quantitation

For the determination of the limit of detection (LoD) and limit of quantitation (LoQ), based on the clinical guideline documents WS/T514-2017 [17] and NCCLS-EP17-A [18], 4 negative serum samples were used as blank samples and tested 15 times within 3 days to get the limit of blank (LoB). Then, 4 serum samples with *Artemisia*-sIgE between 1 LoB and 4 LoB were tested 15 times within 3 days to get LoD. As for LoQ, a serum sample (0.42 kU_A/L) was diluted to 0.21 kU_A/L, 0.11 kU_A/L, and 0.06 kU_A/L, and these 4 serum samples were tested 20 times in 1 batch.

Linearity

According to the clinical guideline documents WS/T408-2012 [19] and NCCLS-EP6-A [20], a high positive (97.53 kU_A/L) and negative sera (0.27 kU_A/L) were chosen to determine the range of linearity. Eleven concentrations of samples were prepared, and the highest and lowest concentration samples were untreated high positive and negative sera, respectively. The middle concentration samples were mixtures of high positive and negative sera at a ratio of 1:9, 2:8, 3:7, 4:6, 5:5, 6:4, 7:3, 8:2, and 9:1. Each sample was tested 3 times in 1 batch.

Statistical Analysis

Standard deviation, mean, and coefficient of variation (CV) were calculated by using Microsoft Excel 365 in standard descrip-

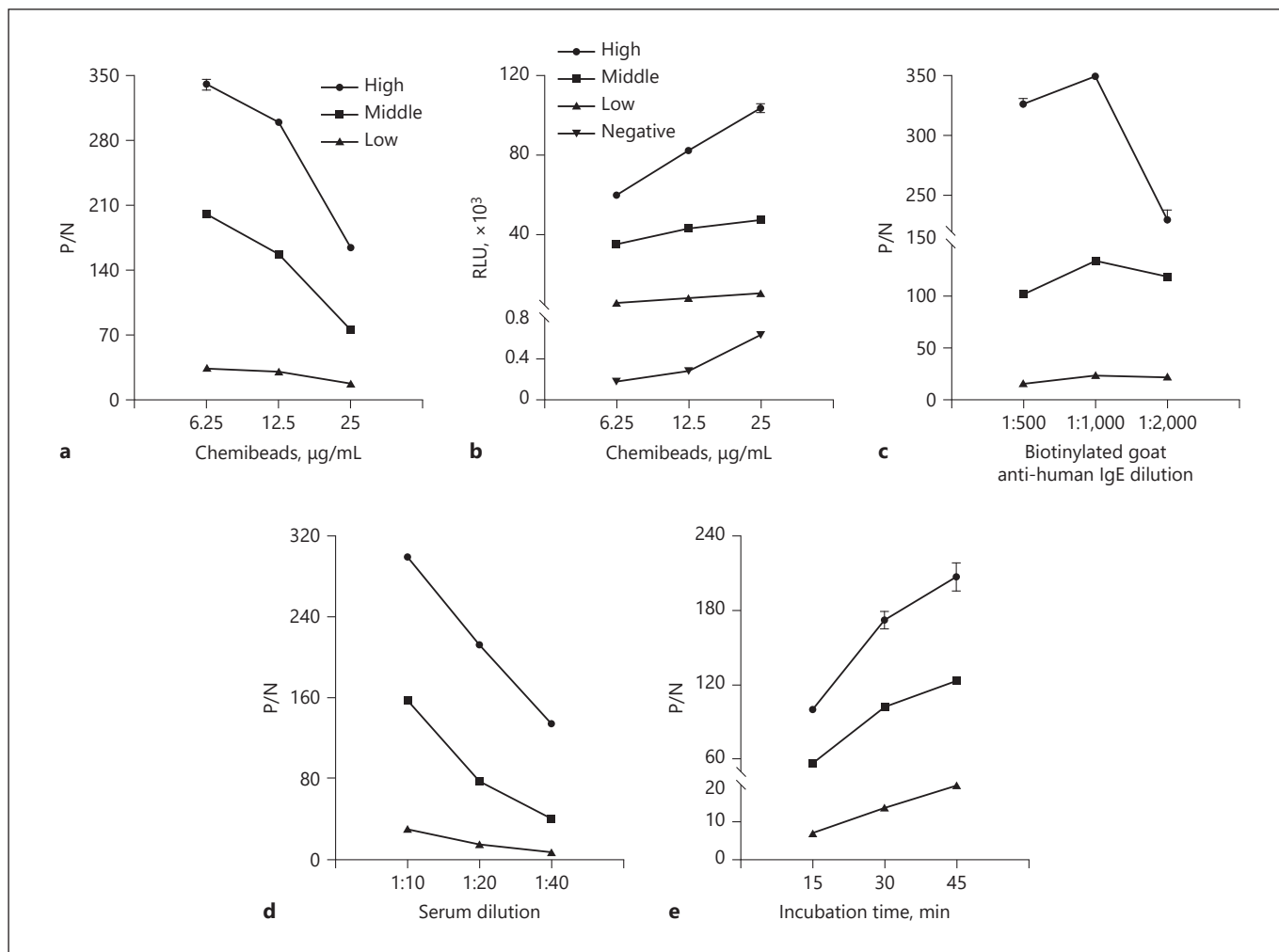


Fig. 2. Optimization of *Artemisia*-sIgE-LICA reaction conditions. **a, b** The effect of different concentrations of *Artemisia*-coated chemibeads. **c** The effect of different concentrations of biotinylated goat anti-human IgE. **d** The effect of different dilutions of serum samples. **e** The effect of different incubation time in the first stage. The 4 lines represent high, medium, and low *Artemisia*-sIgE value in positive serum samples and negative serum samples, respectively. Error bars indicate the range values of measurements. Some error bars are invisible because the magnitudes of them are smaller than the point symbols. P/N, the CL signals ratio of positive (P) to negative sample (N); RLU, relative light unit; LICA, light-initiated chemiluminescence assay.

tive statistical methods. The 4-parameter logistic (4 PL) curve and other graphs were plotted by GraphPad Prism 8. A figure for the principle and procedure of LICA was drawn by biorender (<https://biorender.com/>). ELISACalc was used to calculate the *Artemisia*-sIgE concentration of serum samples.

Results

Optimization of the Reaction Dilution

The concentration of *Artemisia*-coated chemibeads mainly determined the background, and the concentra-

tion of biotinylated goat anti-human IgE mainly determined the CL signals. The CL signals ratio of positive (P) to negative sample (N; P/N) represents the signal-to-noise ratio, which shows the difference in CL signals between positive and negative samples. We looked forward to obtaining low CL signals for negative samples and high CL signals for positive samples, which means a high P/N ratio. Although the P/N ratio declined with an increase in the concentration of *Artemisia*-coated chemibeads (shown in Fig. 2a), the CL signals increased (shown in Fig. 2b). In addition, when the concentration of chemi-

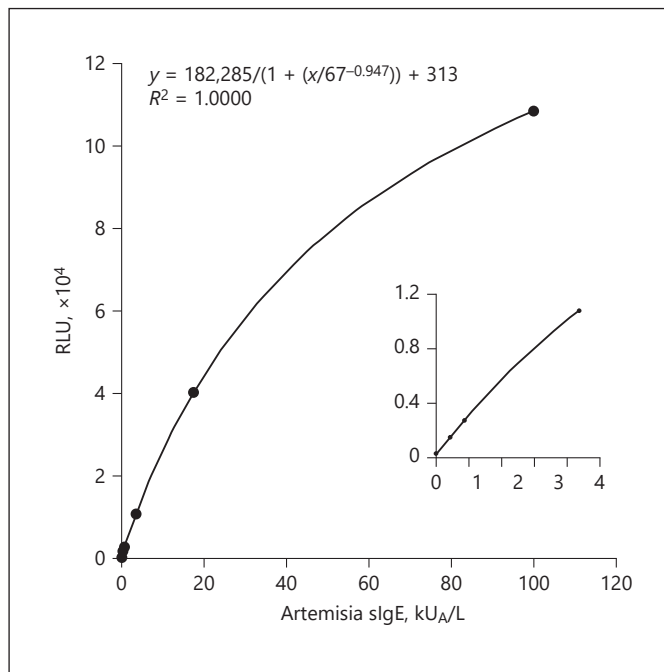


Fig. 3. Calibration curve of LICA on quantitating *Artemisia*-sIgE in human serum. Each point represents the mean value of replicate measurements ($n = 2$). Error bars represent standard deviations. R^2 , determination coefficient; LICA, light-initiated chemiluminescence assay.

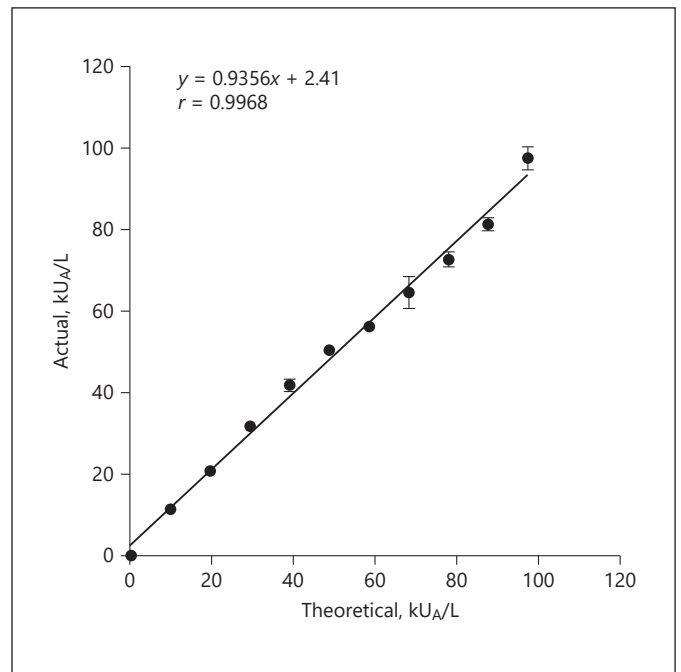


Fig. 4. The range of linearity for LICA on quantitating *Artemisia*-sIgE in human serum. Each point represents the mean value of replicate measurements ($n = 3$). r , correlation coefficient; LICA, light-initiated chemiluminescence assay.

Table 1. The repeatability and intermediate precision of LICA for quantitation of *Artemisia*-specific IgE

Samples	Repeatability ($n = 20$)			Intermediate precision ($n = 40$)		
	mean, kU _A /L	SD	% CV	mean, kU _A /L	SD	% CV
High	78.27	2.50	3.20	78.42	3.37	4.30
Medium	22.74	0.49	2.14	21.78	0.87	4.00
Low	0.42	0.02	3.85	0.41	0.02	4.40

LICA, light-initiated chemiluminescence assay; SD, standard deviation; CV, coefficient of variation.

beads was 6.25 µg/mL, the CL signals of negative samples dropped below 200, which was below the detection capabilities of the machine (shown in Fig. 2b). However, both the cost and the background increased when the concentration of chemibeads was 25 µg/mL. Considering the CL signals and P/N ratio, 12.50 µg/mL was selected as the most appropriate choice. The appropriate dilution of biotinylated goat anti-human IgE could also reduce the cost and improve the CL signals of positive samples. Obviously, the P/N ratio was highest at the dilution of 1:1,000

(shown in Fig. 2c). Consequently, 1:1,000 was the most suitable dilution of the biotinylated goat anti-human IgE.

The results for different dilutions of serum are shown in Figure 2d. When serum dilution was 1:10, the P/N ratio was the highest. Consequently, 1:10 was the most optimal serum dilution. The P/N ratio was at maximum when the first incubation time was 45 min (shown in Fig. 2e), but the P/N ratio did not increase as much in 30–45 min as it did in 15–30 min. Considering that the first incubation time of established egg-sIgE and cow's milk-sIgE-LICA-

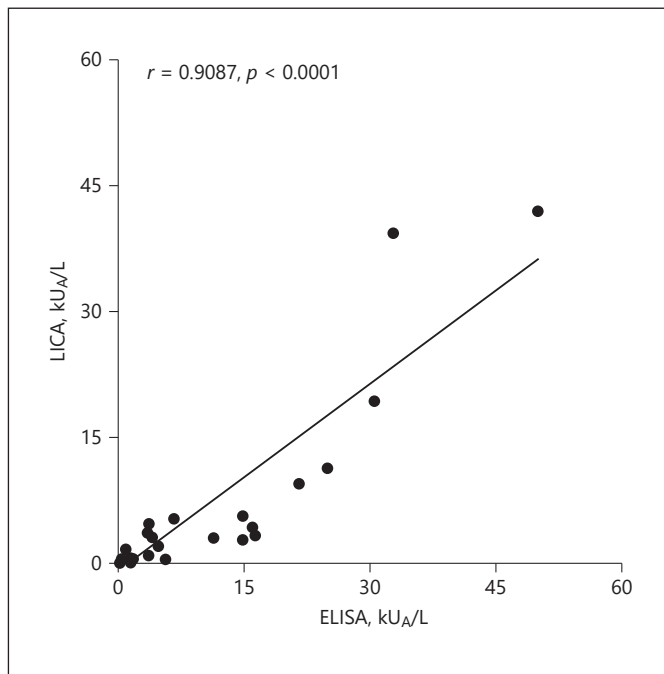


Fig. 5. Correlation between *Artemisia*-sIgE levels in samples measured by LICA and capture ELISA (HOB). LICA, light-initiated chemiluminescence assay.

based detection methods is 30 min, 30 min was selected as the optimal incubation time.

Calibration Curve

As shown in Figure 3, the CL signals and concentrations of 6 calibrators were imported into the GraphPad Prism 8, and then a 4-parameter logistic equation $y = (A - D) / [1 + (x/C)^B] + D$ ($A = 182,598$, $B = -0.947$, $C = 67$, $D = 313$, $R^2 = 1.0000$) was obtained. One calibration curve was prepared in each batch.

Evaluation of Clinical Performance

As shown in Table 1, the CVs for repeatability and intermediate precision were 3.20%, 2.14%, and 3.85% and 4.30%, 4.00%, and 4.40%, respectively. They all were <5%, which implies good precision and repeatability.

LoB (0.03 kU_A/L) was obtained from the results of 4 blank samples according to the equation $LoB = \text{result at position } [N_B (p/100) + 0.5]$ ($p = 95$, $N_B = 60$) (online suppl. Table 1; for all online suppl. material, see www.karger.com/doi/10.1159/000520511). LoD (0.10 kU_A/L) was the median of results from 4 low samples (online suppl. Table 1). LoQ is the lowest actual concentration at which the analyte is reliably detected and at which the uncertainty

Table 2. Method comparison of LICA and capture ELISA (HOB) for detection of *Artemisia*-specific IgE

LICA	ELISA (HOB)		N
	positive, n	negative, n	
Positive	37 (a)	0 (b)	37
Negative	8 (c)	19 (d)	27
Total	45	19	64

LICA, light-initiated chemiluminescence assay.

of the observed test result is less than or equal to the goal set by the laboratory or the manufacturer of the method. As it can be seen in online supplementary Table 2, among the samples with CVs being below 10% that is the goal of this method, 0.11 kU_A/L was the minimum concentration. As a result, LoQ was 0.11 kU_A/L.

Linearity tests were performed to validate and assess the accuracy of LICA. The data were imported into the GraphPad Prism 8, and the linear equation $y = 0.9356 \times X + 2.41$ ($r = 0.9968$) was obtained. As shown in Figure 4, the linearity was over a wide range of 0.27 kU_A/L–97.53 kU_A/L.

Comparison with the Capture ELISA

As shown in Table 2, the sensitivity [$a/(a + c) \times 100\%$] and specificity [$d/(b + d) \times 100\%$] of LICA for the quantitation of *Artemisia*-sIgE are 82.20% and 100%, respectively. The correlation coefficient for regression analysis between the quantitative results of LICA and capture ELISA was 0.9087 (shown in Fig. 5).

Discussion

The genus *Artemisia* is a major outdoor allergen responsible for allergic rhinitis and airway hyperresponsiveness [21]. *Artemisia*-sIgE measurement and clinical history are the cornerstones of *Artemisia* pollen allergy diagnosis. At present, in vitro detection of sIgE has become an important tool for screening for allergic diseases [22], and substantial progress has been made in the development of in vitro sIgE detection assays. The commercial tests of sIgE include EUROLINE (EUROIMMUN), IMMULITE (Siemens) [23], and ImmunoCAP system (Thermo Fisher Scientific). ImmunoCAP is a quantitative method based on a single allergen and/or component attached to a solid phase [24]. As an accurate and quality-

controlled assay, ImmunoCAP is the generally accepted reference for quantitation of sIgE [25]. However, it also needs a large amount of serum and is expensive [26]. As semiquantitative assays, EUROLINE and IMMULITE are more inexpensive than ImmunoCAP but consume more serum and time.

LICA, a highly sensitive, reproducible, and rapid homogeneous assay, is very appropriate for the detection of allergen-sIgE. Therefore, we developed a quantitative LICA-based assay for the detection of *Artemisia*-sIgE, and it is also the first attempt to quantitate inhaled allergen-sIgE by LICA. The reaction of LICA is excited by 680 nm and produces the chemiluminescent emission at 520–620 nm [12]. As the wavelength of readout is significantly different from excitation, it has a low background. The whole experiment of LICA has only 2 routine steps, requires 4 reagents, and does not need to be washed within 1 h. Obviously, it is easily operated and has faster detection rates. Moreover, the mixed proteins of the *Artemisia* pollen are connected to the chemibeads by chemical bonds. Chemibeads have a big specific surface area and can provide more binding sites for allergen proteins, including mixed proteins. Consequently, it could reduce the interference of allergen-specific IgG [13], which will appear in the detection of allergen-sIgE [27, 28]. In China, the reader of LICA is general equipment and exists in numerous clinical laboratories. It could test multiple items, such as thyroid function, sex hormones, and tumor markers. In this way, costs will reduce when several test items share 1 instrument.

This assay is developed to quantify *Artemisia*-sIgE. Accordingly, we used patients' serum with *Artemisia* allergy, whose *Artemisia*-sIgE is high enough to establish the calibration curve. Similar to ImmunoCAP, we will use human total IgE to establish a calibration curve when we develop an LICA-based assay that can quantitate lots of allergen-sIgE in the future. The optimization of LICA made it possible to determine *Artemisia*-sIgE near 0.10 kU_A/L, which allows identifying allergic patients with higher sensitivity (82.20%) and specificity (100%), using only 2.50 µL of serum. This is substantially less than in ImmunoCAP, where 50 µL of serum would be needed for detecting each allergen-sIgE [29]. As a homogeneous immunoassay, this assay has low CVs of repeatability (3.20%, 2.14%, and 3.85%) and intermediate precision (4.30%, 4.00%, and 4.40%), implying a favorable precision. LoD (0.10 kU_A/L) and LoQ (0.11 kU_A/L) set for LICA are close to the reference method ImmunoCAP [29]. In addition, 0.10 kU_A/L and 0.11 kU_A/L are well below 0.35 kU_A/L, which is the serologic medical determination value of al-

lergy, meaning that this assay can accurately distinguish between positive and negative patients for *Artemisia* pollen allergy. There is a statistically significant correlation between the quantitative results obtained from capture ELISA and those obtained from LICA ($r = 0.9087$, $p < 0.0001$). The slight differences in several samples' *Artemisia*-sIgE value may be due to different analytical systems and allergen compositions, which need to be proved by further experiments.

In conclusion, we have developed a quantitative LICA-based assay for the detection of *Artemisia*-sIgE. It has simple operation, rapid detection, and good clinical performance, representing a significant improvement in the clinical sensitivity of the most currently used methods for the diagnosis of *Artemisia* pollen allergy. Consequently, LICA has a great potential for quantification of many other allergen-sIgE. In brief, LICA allows the diagnosis of *Artemisia* pollen allergy with a high predictive value in a faster and easier way.

Acknowledgments

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Statement of Ethics

The study was approved by the Ethics Committee of Tianjin Medical University (TMUHMEC2017008) and conducted by the principles of the World Medical Association Declaration of Helsinki. All patients or caregivers provided written informed consent to participate in the study.

Conflict of Interest Statement

All authors have declared they have no relevant conflicts of interest.

Funding Sources

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Author Contributions

Dandan Liu carried out the experiments and data collection. Lina Zhu, Lisheng Zheng, and Shaoshen Li collected the samples. Bei Zhang and Xin Tan participated in statistical analysis. Yang Yu and Huiqiang Li conceived the study and assisted in data interpretation. All authors reviewed, edited, and approved the final manuscript.

Data Availability Statement

All data generated or analyzed during this study are included in this article and its online supplementary material files. Further enquiries can be directed to the corresponding author.

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Supplementary Material

Table S1

The results for Artemisia-specific IgE of four blank samples and four low samples that were used to determine the limit of blank and limit of detection.

	LoB(kU _A /L)				LoD(kU _A /L)			
	B1	B2	B3	B4	L1	L2	L3	L4
Day 1	0.000	0.027	0.030	0.002	0.104	0.043	0.124	0.101
	0.000	0.005	0.031	0.027	0.089	0.044	0.116	0.101
	0.008	0.022	0.025	0.029	0.095	0.049	0.113	0.095
	0.002	0.025	0.025	0.024	0.098	0.047	0.129	0.112
	0.000	0.015	0.033	0.010	0.101	0.047	0.119	0.109
Day 2	0.000	0.031	0.029	0.001	0.101	0.048	0.129	0.096
	0.000	0.005	0.023	0.014	0.102	0.050	0.120	0.110
	0.000	0.009	0.018	0.000	0.097	0.040	0.111	0.100
	0.000	0.004	0.021	0.012	0.084	0.050	0.117	0.116
	0.000	0.016	0.018	0.012	0.118	0.039	0.123	0.108
Day 3	0.000	0.010	0.023	0.002	0.092	0.042	0.126	0.092
	0.001	0.013	0.021	0.003	0.095	0.049	0.123	0.107
	0.000	0.011	0.018	0.008	0.105	0.047	0.114	0.110
	0.000	0.016	0.013	0.000	0.112	0.035	0.127	0.109
	0.000	0.024	0.031	0.000	0.097	0.042	0.115	0.108

B1, B2, B3, B4 represent four blank serum samples respectively; L1, L2, L3, L4 represent four low serum samples respectively.

Table S2

The results for Artemisia-specific IgE of one sample and its three diluted samples that were used to determine the limit of quantitation.

Samples(kU _A /L)	Mean(kU _A /L)	SD	%CV
0.42	0.416	0.016	3.814
0.21	0.205	0.015	7.317
0.11	0.125	0.007	5.600
0.05	0.085	0.011	12.941

SD: standard deviation, CV: coefficient of variation.