

# Determination of specific autoantibodies in patients with systemic lupus erythematosus by Line immunoassay, ELISA and CLIF assay

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## Abstract

**Background:** Detection of specific antinuclear-antibodies is very importance in term of diagnosis, prognosis and management of patients with systemic lupus erythematosus (SLE). To date, Line immunoassay (LIA), enzyme-linked immunosorbent assay (ELISA) and *Crithidia luciliae* indirect immunofluorescence (CLIF) assay are commonly used for detection of specific antinuclear-antibodies.

**Objective:** To determine the performance of LIA, ELISA and CLIF for the detection of anti-double-stranded DNA (dsDNA), anti-nucleosome, and anti-extractable nuclear antigens (ENA) antibodies in patients with SLE.

**Methods:** A total 100 sera from 50 patients with SLE, 25 patients with disease control and 25 healthy control subjects were tested for anti-dsDNA, anti-nucleosome, and anti-ENA antibodies by LIA, ELISA, and CLIF assay. Agreement and diagnostic performance of each assay were analyzed using Cohen's kappa coefficient and receiver operating characteristic curve analysis.

**Results:** For the detection of anti-dsDNA antibody, ELISA had a substantial agreement with CLIF assay ( $\kappa = 0.74$ ) but LIA had a fair agreement with ELISA and CLIF assay ( $\kappa = 0.37$ , and  $0.35$  respectively). For the detection of anti-nucleosome, anti-nRNP/Sm, anti-Sm, anti-SSA, and anti-SSB antibodies, LIA had a substantial to perfect agreement with ELISA ( $\kappa = 0.64, 0.78, 0.68, 0.91$ , and  $0.74$ , respectively). Anti-dsDNA-NcX ELISA and anti-dsDNA CLIF assay had equally diagnostic performance (sensitivity, 66% vs. 68%, and specificity, 96% vs. 94%, respectively) whereas, anti-dsDNA LIA has low sensitivity (22%) but high specificity (100%).

**Conclusion:** LIA, ELISA, and CLIF demonstrated comparable performance for the detection of specific antinuclear-antibodies. However, there were some discrepancy between assays particularly in the detection of anti-dsDNA antibody.

**Key words:** Systemic lupus erythematosus (SLE), Specific antinuclear-antibodies, Line immunoassay (LIA), Enzyme-linked immunosorbent assay (ELISA), *Crithidia luciliae* indirect immunofluorescence (CLIF)

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## Introduction

Systemic lupus erythematosus (SLE) is a heterogeneous autoimmune disease affecting multiple organ systems.<sup>1</sup> The hallmark of the disease is the production of several auto-antibodies against wide range of nuclear antigens, which have been reported over 100 different antinuclear antibodies (ANA).<sup>2,3</sup>

Some auto-antibodies habitually associated with clinical feature or disease activity. Nowadays, some of these ANA, especially antibody against native double strand DNA (anti-dsDNA), nucleosome (anti-nucleosome) and extractable nuclear antigen (anti-ENA), are generally used in clinical practice as diagnostic and prognostic biomarkers, allowing clinicians to accurately diagnose, monitor the disease activity, and predict the clinical manifestations of patients with SLE.<sup>4-6</sup> Moreover, anti-dsDNA are also emphasized in research area by being implemented in the current classification criteria and the disease activity index of SLE.<sup>7-12</sup>

To date, various commercial analytic system for ANA detection have been developed. Line immunoassay (LIA), enzyme-linked immunosorbent assay (ELISA) and *Crithidia luciliae* indirect immunofluorescence (CLIF) are widely used for anti-dsDNA, anti-nucleosome and anti-ENA detection of in patients with SLE. Each determination system has some differences including single or multiple autoantibody detection, quantitative or semi-quantitative system. For example, LIA and CLIF are semi-quantitative system whereas, ELISA is quantitative system. Up till now, several ELISA and multiplex LIA assays for anti-dsDNA, anti-nucleosome and anti-ENA determination have been continuously developed. For example, ELISA for anti-dsDNA-loaded nucleosomes (dsDNA-NcX) determination has been established recently, to increase its diagnostic performance. Multiplex LIA assays are frequently used due to their advantages such as the necessity of only little amount of sera, time saving, the simultaneous detection multiple auto-antibodies from a single assay and no automate machine requirement. Despite their advantages, there has been growing concern about the equivalent of result among assays.<sup>6,13-17</sup> Previous studies reported that the performance of LIA differs significantly relying on the manufacturer or supplies. Nevertheless, these assays have never been directly compared in the same study. Therefore, the aim of our study is to determine the agreement among LIA, ELISA, and CLIF assay for the detection of anti-dsDNA, anti-nucleosome, and anti-ENA antibodies in patients with SLE, as well as its diagnostic and prognostic performance.

## Patients and Methods

### Patients and controls

The study was conducted at Rheumatology clinic at King Chulalongkorn Memorial Hospital, Bangkok, Thailand, between September 2015 and February 2017. A total of 100 subjects were enrolled which categorized into 3 groups. Group 1 (SLE group) consisted of 50 patients with SLE, diagnosed according to the 1997 ACR classification criteria for SLE but excluded the anti-dsDNA antibody component.<sup>7,8</sup> Group 2 (disease control group) consisted of 25 patients with non-SLE systemic autoimmune rheumatic diseases (non-SLE SARDs); patients with primary Sjögren's syndrome (SS), diagnosed according to the 2002 AECG revised classification criteria for SS (n = 7),<sup>18</sup> patients with diffuse cutaneous systemic sclerosis (SSc, diagnosed according to the 2013 ACR/EULAR classification criteria for SSc (n = 7),<sup>19</sup> patients with polymyositis and dermatomyositis, diagnosed according to the Bohan and Peter criteria (n = 7),<sup>20</sup> and patients with rheumatoid arthritis (RA),

diagnosed according to the 2010 ACR/EULAR classification criteria for RA (n = 4).<sup>21</sup> Group 3 (healthy control group) consisted of 25 age- and sex-matched healthy blood donors whose were recruited from the National Blood Center, Thai Red Cross Society. All SARDs patients were excluded if they had features of overlap syndrome, defined as fulfilled more than one established classification criteria of SARDs, active infection requiring systemic antimicrobial agent use, history of malignancy, pregnancy or lactation and a history of sulfasalazine and TNF inhibitor use. The study was approved by the ethical committee of the King Chulalongkorn Memorial Hospital, Chulalongkorn University. All participants provided informed consent.

At the enrollment, demographic and clinical data including age, gender, disease duration, fulfillment of classification criteria, cumulative clinical manifestations, disease activity using the Mex-SLEDAI score (for the purpose to exclude the anti-dsDNA antibody component to avoid bias),<sup>22,23</sup> and current medication use were collected. The baseline characteristics of the patients and controls are shown in **Table 1 and 2**.

**Table 1. Demographic data of the patients and controls**

	Group 1 SLE n = 50 (Mean ± SD)	Group 2 disease control n = 25 (Mean ± SD)	Group 3 healthy control n = 25 (Mean ± SD)
Age at enrollment (years)	33.8 ± 11.7	51.1 ± 8.9	33.2 ± 10.8
Age at diagnosis (years)	26.2 ± 10.8	45.1 ± 11.3	-
Disease duration (years)	7.7 ± 8.2	6.0 ± 5.9	-
Female: male ratio	47:3	20:5	23:2

**Abbreviation:** SD, Standard deviation

**Table 2. Baseline characteristics of the patients**

Number of fulfilled ACR criteria, excluded the anti-dsDNA antibody component (Mean ± SD)	5.5 ± 1.4 (4-9)
The Mex-SLEDAI score (Mean ± SD)	4.6 ± 5.9 (0-22)
<b>Current immunosuppressive drugs</b>	<b>n (%), dosage (Mean ± SD)</b>
Total	48 (96)
Antimalarial treatment (mg/day)	39 (78)
Hydroxychloroquine (mg/day)	33 (66), 212.9 ± 120.6
Chloroquine (mg/day)	6 (12), 187.50 ± 68.5
Prednisolone (mg/day)	47 (94), 17.8 ± 16.2
Intravenous cyclophosphamide (mg/day)	4 (8), 925.0 ± 398.6
Mycophenolate mofetil (mg/day)	14 (28), 2142.8 ± 886.4
Azathioprine (mg/day)	6 (12), 66.7 ± 30.3
Methotrexate (mg/week)	5 (10), 8.5 ± 2.2
Others	5 (10)

Table 2. (Continued)

Cumulative clinical manifestations	n (%)
Mucocutaneous manifestations	50 (100)
Malar rash	23 (46)
Discoid rash	33 (66)
Photosensitivity	21 (42)
Oral ulcer	21 (42)
Nonscarring alopecia	31 (62)
Cutaneous vasculitis	8 (16)
Livedo reticularis	6 (12)
Hematologic manifestations	35 (70)
AIHA	17 (34)
Leukopenia	21 (42)
Lymphopenia	21 (42)
Thrombocytopenia	11 (22)
APS	4 (8)
Lupus nephritis, number of biopsy-proven	33 (66), 11 (22)
Arthritis	21 (42)
Serositis	12 (24)
Cardiopulmonary manifestations	6 (12)
Neurologic manifestations	6 (12)
Retinal vasculitis	2 (4)
Raynaud's phenomenon	14 (28)
Lymphadenopathy	11 (22)
Constitutional symptoms	22 (44)

**Abbreviation:** AIHA, Autoimmune hemolytic anemia; APS, Antiphospholipid Antibody Syndrome

### Antibody assays

Serum samples were collected at the visit time, then divided into 3 aliquots and stored at  $-20^{\circ}\text{C}$  until use. The anti-RNP/Sm, anti-Sm, anti-SS-A, anti-SS-B and anti-nucleosomes were analyzed using LIA (ANA profile 1, EUROIMMUN Medizinische Labordiagnostika AG.), ELISA (EUROIMMUN Medizinische Labordiagnostika AG.) The anti-dsDNA determination was analyzed using anti-dsDNA in ANA profile 1 (EUROIMMUN Medizinische Labordiagnostika AG.), anti-dsDNA-NcX ELISA (EUROIMMUN Medizinische Labordiagnostika AG.) and CLIF assay (*Crithidia luciliae* sensitive IIFT, EUROIMMUN Medizinische Labordiagnostika AG.) All assay procedures were performed following the manufacturer's instructions. The results were then evaluated using the EUROLineScan software. All of the results were interpreted according to manufacturer's cut-off.

### Statistical assays

The agreement between LIA, ELISA, and CLIF assay were assessed by Cohen's kappa coefficient and agreement rate. The diagnostic performances of the assays were assessed by sensitivity, specificity, receiver operating characteristic (ROC) curve analysis, and the areas under the curve (AUC). All Statistical analyses were performed using SPSS for Windows, version 22.0 (SPSS Inc., Chicago, IL, USA).

## Results

### Comparison of assays for the detection of anti-dsDNA and anti-nucleosome antibody

The agreement rate between anti-dsDNA LIA and anti-dsDNA-NcX ELISA, anti-dsDNA LIA and anti-dsDNA CLIF assay, anti-dsDNA-NcX ELISA and anti-dsDNA CLIF assay were 76%, 74%, and 88% respectively. The Cohen's kappa coefficient between anti-dsDNA LIA and anti-dsDNA-NcX ELISA, anti-dsDNA LIA and anti-dsDNA CLIF assay were fair;  $\kappa = 0.37$ , and  $0.35$  respectively. In contrast, the Cohen's kappa coefficient between anti-dsDNA-NcX ELISA and anti-dsDNA CLIF assay was substantial;  $\kappa = 0.74$ . The discrepancy results are shown in Table 3.

Table 3. Comparison of assay agreement for the detection of anti-dsDNA, anti-nucleosome, and anti-ENA antibody.

Antibodies and assays	Cohen's Kappa* (95% CI)	Agreement (%)	+/+	+/-	-/+	-/-
Anti-dsDNA						
LIA vs ELISA	0.37 (0.20-0.54)	76	11	0	24	65
LIA vs CLIF	0.35 (0.19-0.51)	74	11	0	26	63
ELISA vs CLIF	0.74 (0.60-0.88)	88	30	5	7	58
Anti-nucleosome						
LIA vs ELISA	0.65 (0.48-0.83)	87	17	3	10	70
Anti-nRNP/Sm						
LIA vs ELISA	0.78 (0.65-0.91)	90	30	6	4	60

**Abbreviation:** LIA, Line immunoassay; ELISA, enzyme-linked immunosorbent assay; CLIF, *Crithidia luciliae* indirect immunofluorescence assay; Anti-dsDNA, double-stranded DNA antibody; Anti-nRNP/Sm, n ribonucleoprotein/Smith antibody; Anti-SSA, Ro cytoplasmic RNA protein antibody; Anti-SSB, La cytoplasmic RNA protein antibody

Table 3. (Continued)

Antibodies and assays	Cohen's Kappa* (95% CI)	Agreement (%)	+/+	+/-	-/+	-/-
<b>Anti-Sm</b>						
LIA vs ELISA	0.68 (0.46-0.90)	93	9	3	4	84
<b>Anti-SSA</b>						
LIA vs ELISA	0.91 (0.83-0.99)	96	34	2	2	62
<b>Anti-SSB</b>						
LIA vs ELISA	0.74 (0.40-1.00)	98	3	2	0	95
<b>Anti-dsDNA vs Anti-nucleosome</b>						
ELISA vs LIA	0.59 (0.42-0.75)	83%	19	16	1	64
ELISA vs ELISA	0.88 (0.77-0.98)	92%	27	8	0	65

**Abbreviation:** LIA, Line immunoassay; ELISA, enzyme-linked immunosorbent assay; CLIF, *Crithidia luciliae* indirect immunofluorescence assay; Anti-dsDNA, double-stranded DNA antibody; Anti-nRNP/Sm, n ribonucleoprotein/Smith antibody; Anti-SSA, Ro cytoplasmic RNA protein antibody; Anti-SSB, La cytoplasmic RNA protein antibody

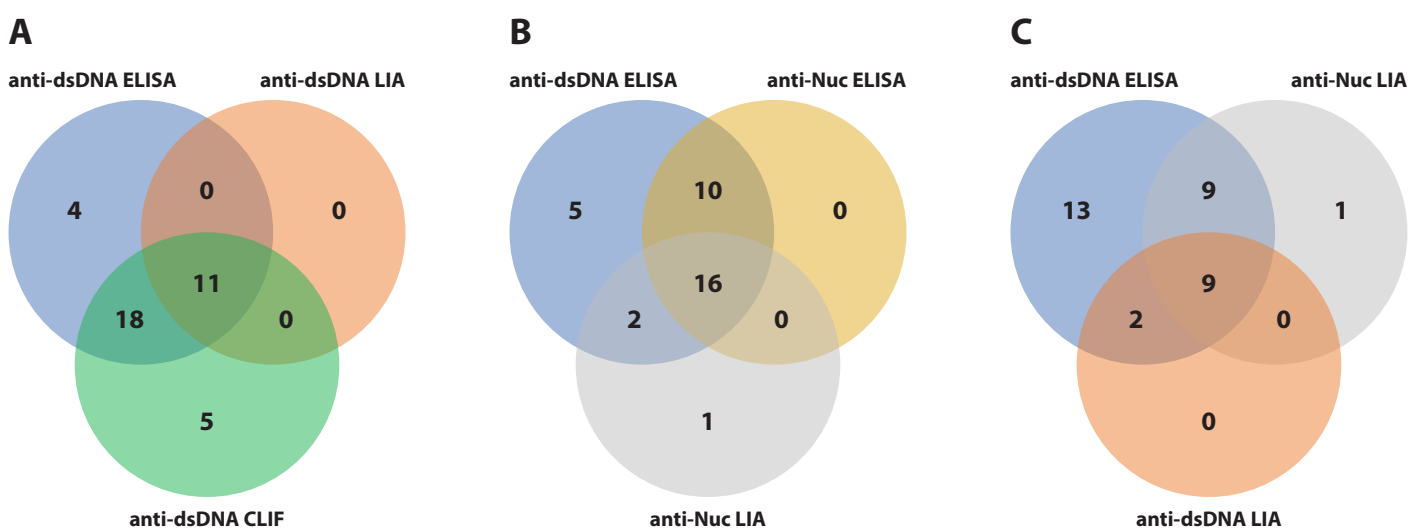
The agreement rate between anti-nucleosome LIA and anti-nucleosome ELISA, anti-nucleosome LIA and anti-dsDNA-NcX ELISA, anti-nucleosome ELISA and anti-dsDNA-NcX ELISA were 87%, 83, and 92% respectively. The Cohen's kappa coefficient between the assays were moderate to perfect;  $\kappa = 0.64, 0.59, \text{ and } 0.81$  respectively. The discrepancy results are shown in **Table 3**.

In comparison of patients with SLE who were positive for anti-dsDNA antibody by at least one assay, of those 38 patients, 33 (87%) were positive with anti-dsDNA-NcX ELISA, 34 (89%) were positive with anti-dsDNA CLIF assay, and 11 (29%) were positive with anti-dsDNA LIA. Interestingly, all SLE patients who were positive anti-dsDNA by LIA were also positive anti-dsDNA-NcX ELISA and anti-dsDNA CLIF assay.

In addition, all SLE patients who positive anti-nucleosome ELISA were also positive anti-dsDNA-NcX ELISA. Moreover, of those 22 SLE patients with positive anti-dsDNA-NcX ELISA but negative anti-dsDNA LIA, 18 (82%) were positive either anti-nucleosome LIA or anti-nucleosome ELISA. (**Figure 1**)

**Comparison of assays for the detection of anti-ENA antibodies**

All assays displayed comparable in detection of anti-ENA antibodies. The agreement rate between anti-nRNP/Sm LIA and ELISA, anti-Sm LIA and ELISA, anti-SSA LIA and ELISA, and anti-SSB LIA and ELISA were 90%, 93%, 96%, and 98% respectively. The Cohen's kappa coefficient between the assays were substantial to perfect;  $\kappa = 0.78, 0.68, 0.91, \text{ and } 0.74$  respectively. The discrepancy results are shown in **Table 3**.



**Figure 1.** The Venn diagram of the overlapping of the positivity of anti-dsDNA and anti-nucleosome in the patients with SLE (n = 50) by LIA, ELISA, and CLIF assays. (A) Numbers of SLE patients with positive the anti-dsDNA-NcX ELISA, anti-dsDNA LIA, and anti-dsDNA CLIF. (B) Number of SLE patients with positive the anti-dsDNA-NcX ELISA, anti-nucleosome LIA, and anti-nucleosome ELISA. (c) Number of SLE patients with positive the anti-dsDNA-NcX ELISA, anti-dsDNA LIA and anti-nucleosome LIA.

**Abbreviation:** Anti-dsDNA, double-stranded DNA antibody; Anti-dsDNA-NcX, double-stranded DNA loaded nucleosome antibody

### Diagnostic and Prognostic performance

The diagnostic performances of the assays for detection of anti-dsDNA, anti-nucleosome, and anti-Smith antibodies were assessed in patients with SLE (group 1) and controls (group 2, disease control and group 3, healthy control). The sensitivity, specificity, ROC curves analysis, and the AUC of all assays are shown in **Table 4**. Overall, all assays yielded comparable specificity (94%-100%). Anti-dsDNA CLIF assay revealed highest sensitivity (68%), followed by anti-dsDNA-NcX ELISA (66%), whereas, the anti-dsDNA LIA demonstrated the lowest sensitivity (22%). Using ROC curve analysis, anti-dsDNA-NcX ELISA yielded better AUC values (0.90; 95% CI 0.84 - 0.97) compared with anti-nucleosome (0.88; 95% CI 0.81 - 0.95), and anti-Smith ELISA (0.73; 95% CI 0.63 - 0.83).

**Table 4. Diagnostic performance of anti-dsDNA, anti-nucleosome, and anti-Smith antibody.**

Antibodies and assays	Sensitivity (%)	Specificity (%)	AUC (95% CI)
Anti-dsDNA LIA	22	100	-
Anti-dsDNA-NcX ELISA	66	96	0.90 (0.84-0.97)
Anti-nucleosome LIA	38	98	-
Anti-nucleosome ELISA	52	98	0.88 (0.81-0.95)
Anti-Smith LIA	22	98	-
Anti-Smith ELISA	22	96	0.73 (0.63-0.83)
Anti-dsDNA CLIF assay	68	94	-

**Abbreviation:** LIA, Line immunoassay; ELISA, enzyme-linked immunosorbent assay; CLIF, *Crithidia luciliae* indirect immunofluorescence assay; Anti-dsDNA, double-stranded DNA antibody; Anti-dsDNA-NcX, double-stranded DNA loaded nucleosome antibody, Anti-Smith, Smith antibody

The disease activity of SLE was evaluated by the Mex-SLE-DAI score. The correlation between autoantibody levels with disease activity of SLE were calculated using Spearman's rho correlation coefficient. Both anti-dsDNA-NcX ELISA and anti-nucleosome ELISA revealed moderate positive correlation;  $R_s = 0.47$ ,  $p = 0.001$  and  $R_s = 0.46$ ,  $p = 0.003$  respectively. Moreover, active disease group had the significant higher level of anti-dsDNA-NcX ELISA and anti-nucleosome ELISA compared to non-active group (mean  $\pm$  SD of anti-dsDNA-NcX ELISA 403.69  $\pm$  293.61 IU/mL vs. 170.67  $\pm$  207.12 IU/mL,  $p = 0.003$ ; mean  $\pm$  SD of anti-nucleosome ELISA 95.02  $\pm$  76.92 IU/mL and 26.48  $\pm$  44.89 IU/mL,  $p < 0.0001$  respectively)

### Discussion

Detection of auto-antibodies is one of the important evidences for diagnosis and management of SLE and other SARDs. At present, a number of autoantibodies determination systems are accessible, however, the issues of assay variability

and reproducibility has been raised. Each of them uses different detection systems, antigen source and preparation, coating system and calibration system. Therefore, the results of autoantibody detected by one system may not identified by the others. In this study, we aim to compare the performance of LIA to ELISA and CLIF assay in detection of autoantibodies in patients with established SLE. Our study showed that LIA and ELISA demonstrated a nearly perfect agreement in detecting almost autoantibodies, especially anti-RNP/Smith, anti-Smith, anti-SS-A, anti-SS-B and anti-nucleosomes. However, LIA showed only a fair agreement with anti-dsDNA-NcX ELISA and anti-dsDNA CLIF assay in detection of anti-dsDNA. The very low sensitivity but extremely high specificity of anti-dsDNA LIA might be a result of different antigen source and preparation and coating system of anti-dsDNA LIA from anti-dsDNA-NcX ELISA and anti-dsDNA CLIF assay.<sup>24</sup> Our study also shown that both anti-dsDNA-NcX ELISA and anti-dsDNA CLIF assay had much higher sensitivity compared to previous study using fluorescence enzyme immunoassay and CLIF assay from other manufacturer.<sup>15</sup> This finding should be explained by the difference in assays and study population.

Recent studies demonstrated that anti-ds-DNA antibodies are constantly assembled with anti-nucleosome antibodies although, anti-nucleosome antibodies do not always display with to anti-ds-DNA antibodies. By ROC analysis, our result showed that anti-dsDNA-NcX ELISA had slightly better diagnostic performance compared to anti-dsDNA CLIF assay and much better diagnostic performance compared to anti-dsDNA LIA in patients with SLE. These results were consistent with previous reports which might be explained by the use of dsDNA-loaded nucleosomes as antigen in this commercial ELISA kit.<sup>25,26</sup> Due to, both anti-dsDNA and anti-nucleosome antibodies have been recommended for monitoring lupus disease activity.<sup>25,26</sup> We analyzed the correlation between anti-dsDNA-NcX ELISA, antinucleosome and the severity of SLE using Spearman's rho correlation coefficient. The results revealed a significant positive correlation between both anti-dsDNA-NcX ELISA and antinucleosome ELISA with Mex-SLE-DAI score in patients with SLE. Our finding emphasized the previous reports that anti-dsDNA and anti-nucleosome antibodies can be used as biomarker for lupus disease severity.<sup>25,26</sup> However, our study had some limitations. Firstly, our study had a relatively small sample size. Secondly, we could not evaluate whether both assays are beneficial for monitoring disease activity over time, as, our study is cross-sectional study. Nonetheless, we recruited the patients with SLE both in active and non-active disease in equal proportions to minimize this limitation.

LIA is a system which is capable to simultaneously detection of multiple antibodies from a single testing, and is currently available in fully automated computer-assisted analysis and interpretation system. Nonetheless, this assay generates some discrepancies among different types of the kits and the manufacturers, which might be responsible for misinterpretations and unnecessary use of consequent diagnostic investigations and treatments.<sup>14,24,27</sup>

## Conclusion

To our knowledge, this study was the first to evaluate the performance of multiple assays from one manufacturer for the detection of anti-dsDNA, anti-nucleosome, and anti-ENA antibodies in patients with SLE. Our results demonstrated the comparable performances among the three assays in the detection of anti-RNP/Sm, anti-Sm, anti-SS-A, anti-SS-B and anti-nucleosome. We also found that anti-dsDNA-NcX ELISA and anti-nucleosome ELISA were positively correlated with Mex-SLEDAI score in patients with SLE. However, to detecting anti-dsDNA by LIA yielded the least sensitivity comparing ELISA and CLIF assays.

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## Disclosures

The authors have declared no conflicts of interest.

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