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Validation of Luminex immunological and competitive Luminex immunological assays for clinical immunogenicity assessment of a 14-valent recombinant human papillomavirus vaccine

Xiao Zhang[1] | Dan Meng[1] | Hong Li[2] | Xuefeng Li[1] | Jing Li[1] | Ping Hu[1] | Lu Zhao[1] | Rui Wang[1] | Chaonan Zhao[1] | Chunxia Luo[1] | Weihua Gu[2] | Wenlin Gai[1] | Yang Wang[1] | Liangzhi Xie[1,3,4]

[1]Beijing Engineering Research Center of Protein and Antibody, Sinocelltech Ltd., Beijing, China

[2]Shanghai Xihua Scientific Co., Ltd., Shanghai, China

[3]Beijing Key Laboratory of Monoclonal Antibody Research and Development, Sino Biological Inc., Beijing, China

[4]Cell Culture Engineering Center, Chinese Academy of Medical Sciences & Peking Union Medical College, Beijing, China

Correspondence

Liangzhi Xie, Sinocelltech Ltd., No.31 Kechuang 7th St, BDA, Beijing 100176, China.
Email: lx@sinocelltech.com

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Abstract

A novel virus-like particle (VLP)-based multivalent recombinant human papillomavirus (HPV) vaccine was developed and evaluated in human, including 14 HPV-type specific VLP antigens (HPV6, 11, 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, and 59). The pseudovirus-based neutralizing assay (PBNA) method is widely used for immunogenicity assessment of HPV vaccine in clinical trials. However, as many as 14 antigen-specific antibody levels need be determined, PBNA is, for many reasons, challenging and time-consuming. In this study, we developed a Luminex immunological assay (LIA) and a competitive Luminex immunological assay (cLIA). These methods increase the throughput, reproducibility and precision, as well as reduce the complexity. All assay parameters showed good characteristics in the validation of both methods, benefiting from highly purified and structurally correct VLPs, high specific antibodies, standard VLP-microspheres and PE-mAbs conjugating process, adequate assay development and stable system. Validation data support the use of both methods for immunogenicity assessment in clinical trials. LIA showed higher sensitivity than cLIA, and due to limited epitopes of mAb, cLIA detected lower antibody responses, and therefore, fewer antibodies. This work not only supports clinical trials of 14-valent HPV vaccines more efficiently and reliably, but also provides a set of validation strategies and usable standards for general vaccine immunogenicity testing.

KEYWORDS

competitive Luminex immunological assay (cLIA), HPV, human papillomavirus, immunogenicity, Luminex immunological assay (LIA), neutralizing antibody, vaccine, VLP

Xiao Zhang and Dan Meng contributed equally to this study.

1 | INTRODUCTION

Human papillomavirus (HPV) as a nonenveloped DNA virus infects skin or mucosal cells. HPV infection commonly causes skin and mucous membrane growths (warts), and is directly linked to cervical cancer. There are more than 100 genotypes of HPV, depending on the organ infected and degree of risk, at least 13 of which are considered high-risk types, can cause cervical cancer[1] and are associated with other anogenital cancers and cancers of the head and neck.[2] HPV16 and 18 as "high-risk" genotypes are responsible for approximately 70% of all cervical cancers and nearly 50% of high grade cervical precancers.[1] According to the statistics of the World Health Organization, there were 604 000 new cervical-cancer cases and 342 000 related deaths in 2020 around the world.[3] Vaccination is a highly cost-effective way to prevent cervical cancer.

As of September 2022, six HPV vaccines with different valences were available in the market which are all L1-protein based virus-like particle (VLP) vaccines, Gardasil® (approved in 2006),[4] Cervarix® (approved in 2007),[5] Gardasil 9® (approved in 2014),[6] Cecolin® (approved in China in 2019),[7] Walrinvax® (approved in China in 2022),[8] and Cervavac®(approved in India in 2022).[9] Among all marketing HPV vaccines, Gardasil 9® covered most extensive HPV types of HPV-related cervical cancer at almost 89.7% worldwide,[10] including carcinogenic types (HPV16, 18, 31, 33, 45, 52, and 58) and genital warts related types (HPV6 and 11).

There is a new VLP-based multivalent recombinant HPV vaccine developed by Sinocelltech, Ltd, which includes 14 specific HPV-VLP antigens (HPV6, 11, 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, and 59), in which 5 novel antigens (HPV 35, 39, 51, 56, and 59) were added to the 9 types overlapping with Gardasil 9®. The vaccine covers all 12 types of high-risk cancer-causing HPV classified by WHO, and has wider protection range against HPV -related cervical cancer than Gardasil 9®, up to 95.4%,[10] resulting in about 55% reduction in potential remaining risk of HPV-related cervical cancer in comparison with Gardasil 9®. The vaccine used the same expression system as Cervarix® and Flublock® to transfect baculovirus infected insect cell. The 14-valent HPV vaccine has been studied in phase II.

During the development of HPV vaccines, neutralizing antibodies are considered to be the main mediator of protection through immunogenicity evaluation, so good immunoassays are essential for clinical trials of HPV vaccines.

To detect *anti*-HPV antibody for evaluating the immunogenicity of vaccine candidates, many assays have been developed and reported, mainly including pseudovirus-based neutralization assays (PBNA), direct binding IgG assay (ELISA and Luminex-based immunoassay, LIA),[11] and competitive Luminex immunoassays (cLIA). All methods can be used to measure HPV specific antibody levels in serum samples. An available assay used for multivalent HPV vaccines needs to be sensitive, reproducible, simple to perform, and capable of high-throughput testing. PBNA has been designated as "the gold standard" by WHO and is recognized and accepted by regulatory agencies for the assessment of HPV immunogenicity.[12] However, with the continuous increase of vaccine valency, immunogenicity evaluation faces many challenges, and the workload is also increasing. PBNA method is complicated to operate with cell-culture requirement, low throughput and high cost, especially for the immunogenicity detection of multivalent HPV vaccines. HPV type specific total binding IgG can be detected by ELISA or LIA methods. Glaxo Smith Kline (GSK) assessed the immunogenicity of Cervarix® in human using a direct-binding ELISA. The assay showed a high correlation with PBNA for HPV16 and HPV18 (correlation coefficients: 0.70–0.94) with human serum samples.[13] Merck assessed the immunogenicity of HPV16 and HPV18 of Gardasil® in humans using cLIA, PBNA, and LIA, respectively. The correlation coefficients for PBNA and LIA for HPV 16 and 18 were 0.95 and 0.93, respectively. cLIA and LIA also showed high correlation coefficients with HPV 16 and 18, both as 0.92. Among cLIA-positive samples, LIA and PBNA tested positive were 100% and 98%, respectively.[14] With the Luminex platform, LIA shows clear advantages over ELISA with multiplexing, higher throughput and sensitivity. LIA can simultaneously detect multivalent antigen-specific IgG with different conjugated magnetic beads in one well, saving time and sample usage. cLIA is also developed based on Luminex platform by Merck.[15] Additionally, the assay employs a panel of HPV neutralizing monoclonal antibodies (mAbs) labeled with phycoerythrin (PE) as competitors. If a neutralizing antibodies that share the same epitope as the mAb is present at proper concentration in the serum sample, the binding of the mAb to the VLP will be inhibited by antibody in the serum. Merck evaluated the immunogenicity of Gardasil®[16–18] and Gardasil 9®[15,19] in human using cLIA and received marketing approval from the FDA and other regulatory agencies. The method has good specificity but can detect lower neutralizing antibodies in samples. Because the assay is limited by the designed or chosen PE-conjugated mAbs which include relatively limited epitopes.[20] In comparison, LIA can detect more specific antibodies. While LIA may detect small amounts of non-neutralizing antibodies, with high-quality HPV-VLP antigen used in detection, neutralizing antibodies in LIA accounted for the majority of HPV-type specific total IgG, as evidenced by the high correlation between direct binding-ELISA and PBNA.

To develop a usable assay for the evaluation of immunogenicity of the 14-valent HPV vaccines, we focused on LIA and cLIA as surrogate candidates for PBNA with support from full method validation. In the nonclinical research phase, we developed the LIA method to successfully detect 14 HPV type-specific total IgG and confirmed good correlation of LIA and PBNA for all 14 HPV types.[13,21]

To meet the analytical needs of immunogenicity assessment in clinical trials, LIA and cLIA have been adapted to the detection of human serum samples from early clinical trial subjects. In this study, the combined validation of LIA and cLIA is described and compared. The biomarker white paper recommends validation of

multiple ligand binding assays, and vaccine trials can be classified as biomarker assays. Finally, our study referred to PK and ADA (*anti*-drug antibody) guidelines as well as vaccines white papers to design a validation protocol for quantitative immunogenicity testing.[22] The study laid a good foundation for the detection of human serum samples. Compared with the reported validation of Gardasil 9® LIA method, the method validation parameters and acceptance criteria designed for 14-valent recombinant vaccine in this study are more specific.

2 | MATERIALS AND METHODS

2.1 | VLP-MS and PE-mAb

Each type of purified HPV VLPs was conjugated with Luminex microspheres (MS). Coupled HPV VLP-MS can be used for sample detection in both LIA and cLIA. A panel of type-specific monoclonal neutralizing antibodies (mAbs or Nabs) was prepared against a single HPV type, which was conjugated with PE (phycoerythrin). The PE-mAb was used as the detection reagents in cLIA. The preparation of critical reagents mentioned above was described in Supporting Information. Each batch of the critical reagents was validated based on established standards as shown in Supporting Information: Tables S2 and S3.

2.2 | Reference sera

Quantitative analysis requires calibration standards. Human sera are difficult to obtain in large amount for human immunogenicity testing and are not mandatory as fixed constant calibration standards. Therefore, the reference serum was prepared in cynomolgus monkeys as discussed in Supporting Information.

Since sera were from monkeys, the standard curve was diluted in different background matrices. The buffer containing 1% goat serum matrix was used as a surrogated substrate to dilute the standard curve samples of the LIA method, and the cLIA method used normal human serum as a blank matrix to dilute the standard curve samples. In both LIA and cLIA methods, all quality control samples and real samples will be diluted with normal human serum.

2.3 | Control serum of normal human

Control serum samples were purchased from the Drug Clinical Trial Center of Huacheng Hospital, and they were collected from females aged 18–45 years. Eligibility criteria required volunteers to be in good health without a history of genital warts or abnormal cervical cytology. Control serum will be used as blank matrix and the negative control for method validation and specific IgG detection for LIA and cLIA.

2.4 | Luminex-based immunoassay (LIA)

Detection of HPV type-specific IgG concentration in human serum based on the LIA method, including HPV6, 11, 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, and 59, is based on equipment developed by Bio-Plex.[21] The Bio-Plex (BIO-RAD) and Luminex® (Luminex) systems perform the same procedures based on the same xMAP technology. The data output with MFI unit for Luminex (Measure Fluorescence Intensity) has a different name from RFI (Relative Fluorescence Intensity) for Bio-Plex, but meaning the same data.

Sample diluent (Phosphate-Buffered Saline with Tween 20% and 1% Bull Serum Albumin, 1% BSA-PBST) was added into black 96-well plate at 50 μL/well and incubated for about 30 min. After empting the 96-well plate, the diluted serum sample was added into the plate at 25 μL/well. The coupled magnetic beads were added into the plate at 25 μL/well (2000 beads/type) and the mixture of serum samples and microspheres were covered and incubated for about 2 h at room temperature for about 2 h to form HPV-IgG complex. Gt F(ab')2 *anti*-human IgG(γ)R-PE Conjugate (ab98596; Abcam) was diluted 1:300 and added to the plate at 50 μL/well as detection reagent. Plates were incubated for approximately 1 h at room temperature in the dark. After washing the plate, 100 μL/well sheath solution was added into the plate to resuspend the magnetic beads on the Bio-Plex 200 device for detection. The RFI value was related to the amount of type-specific total IgG antibodies in the serum bound to the VLPs on the addressable beads. The mechanism of the LIA method is shown in Figure 1A.

2.5 | cLIA

cLIA method was developed based on Bio-Plex system and a panel of neutralizing mAbs that bind to nature epitopes of L1 proteins. Competitive immunoassays detect specific antibodies in human serum that compete for binding to the same epitope of L1 antigen as mAbs.

Competitive assays were performed on black 96-well plates. The plate was wetted with sample diluent 1% BSA-PBST at 200 μL/well and incubated for approximately 30 min. After empting the plate, added diluted serum samples to the plate at 50 μL/well, and with 14 HPV VLPs-conjugated magnetic beads (containing ~2000 magnetic bead per type) and diluted PE-mAbs at 25 μL/well (final concentration of each type was 20 ~ 60 ng/mL). The mixture was incubated overnight at 2 ~ 8°C in the dark. Then, after washing the plate, added 100 μL/well of sheath fluid to resuspend the magnetic beads, ready for detection on the Bio-Plex 200. The RFI value was negatively correlated with the amount of type-specific antibody in the serum for each HPV-VLP conjugated beads. The RFI of all samples was converted into inhibition rate, and negative control samples were used as the basis for comparative evaluation. The mechanism of the cLIA method was shown in Figure 1B.

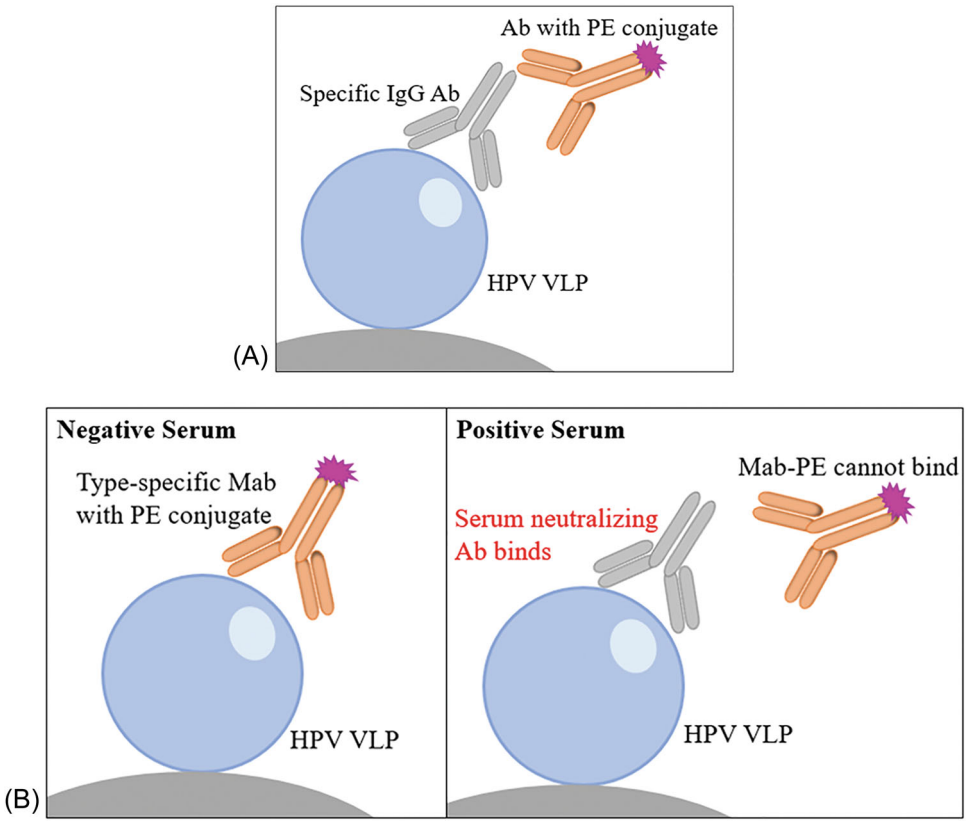

FIGURE 1 Method principle diagram of LIA (A) and cLIA (B). cLIA, competitive Luminex immunological assay; LIA, Luminex immunological assay.

2.6 | Method validation parameters

2.6.1 | Standard curves

For the convenience of calculation, the concentration of each specific antibody or neutralizing antibody in the reference serum was set as 4000 u/mL. The antibody levels were quantified by regression against reference standard sera and reported first in arbitrary unit (u/mL). A mixture of blank serum will be analyzed together with the standards as background signal.

Standard curve samples were serially diluted from reference standard serum. LIA used a seven-point standard curve with twofold serial dilutions of each HPV type ranging from 4 to 200 u/mL. Two u/mL was assigned specifically as the anchor point in enhancing the curve fit. Read the RFI for each point of the standard curve and represent the specific total IgG concentration. The assay used 4-parameter logistic regression (4PL) to fit data from a standard curve to a sigmoid curve. Standard curve concentrations for cLIA ranged from 20 to 200 u/mL (HPV6, 11, 31, and 45) or 10 to 200 u/mL (HPV16, 18, 33, 35, 39, 51, 52, 56, 58, and 59). The inhibition rate of the standard curve samples was calculated according to the RFI of the negative control samples, and then fitted using the same method as LIA.

At least 75% (i.e., at least six different concentration levels) of standard curve samples should meet the quantitative range variable coefficient (%CV) within 25% (lower limit of quantitation [LLOQ] and upper limit of quantitation [ULOQ] were 30%), %Bias was within ±25% (LLOQ and ULOQ were 30%).

2.6.2 | Accuracy and precision

The accuracy and precision of at least six batches were validated by at least two experimenters over a period of at least 2 days. Each batch contained the following samples: one set of freshly prepared standard curve samples, six sets of quality control samples, each set containing five concentration levels of quality control samples and pooled blank human serum: ULOQ, HQC (High Quality Control), MQC (Middle Quality Control), LQC (Low Quality Control), and LLOQ. Pooled blank human serum was added as a negative quality control (NC) sample.

Acceptance criteria for quality control samples (LLOQ, LQC, MQC, and HQC, ULOQ) at each concentration level were within ±25% (30% for LLOQ and ULOQ). The total error of each concentration level must be within 40.0% (50.0% for ULOQ and LLOQ). At least 2/3 of the test batches and 50% of each concentration level should meet the above acceptance criteria.

2.6.3 | Selectivity

Selectivity was assessed using blank matrices from 10 normal individuals. Use each matrix blank as a selective sample to prepare ULOQ, LLOQ, and NC samples. In addition, hemolysis and lipemia are the most frequent endogenous interference that can influence detection results, which need to be assessed in methods validation. Thus, hemolysis (2% v/v hemolysis whole blood) and lipemia (triglyceride concentration of 300 mg/dL) samples were prepared and included in the selectivity assessment with the same criteria.

Acceptance criteria for selective samples (LLOQ and ULOQ) was that %CV does not to exceed 30%, %Bias does not to exceed ±30%, and at least 80% of the matrix-selective samples met the criteria.

2.6.4 | Specificity

To study whether the monovalent HPV vaccine-specific antibody is subjected to other types of HPV interference during the detection process, its specificity was investigated by assessing cross reactivity with adding each type of antigens. QC samples were mixed with single HPV-VLP antigen at 1:1 ratio to obtain specificity samples, with the final concentration of QC samples equal to that of HQC or LQC, and HPV-VLP antigen equal to 45 or 15 μg/mL. Fourteen HPV types, two concentrations of single HPV-VLP antigen and two concentrations of QC samples are mixed separately and then incubated at room temperature for at least 1 h to obtain a testing set with 56 specificity samples. After incubation, in accordance with actual samples storage temperature, the specificity samples were stored for at least additional 12 h. The %CV of specificity samples should not exceed 25%. Results were finally presented in form of recovery rate. If HPV antigen interfered the specific antibody detection, the recovery rate of QC samples will drop dramatically.

2.6.5 | Dilutability

Dilution effect was validated on standard reference dilution samples at 1:20, 1:40, 1:80, 1:200, 1:400, and 1:2000. Samples set above ULOQ were used for hook-effect assessment.

The RFI of samples with theoretical concentrations below the LLOQ should be below the LLOQ of LIA, but the RFI was in different direction for cLIA. %CV should be within 25% (30% for LLOQ and ULOQ), and %Bias should be within ±25% (30% for LLOQ and ULOQ).

2.6.6 | Stability

In stability validation, HQC and LQC samples were introduced under different conditions, including (1) room temperature for 24 h, (2) 2 ~ 8°C for 72 h, (3) having eight consecutive freeze-thaw cycles, (4) −90 to −60°C for 96 days, and (5) −25 ~ −15°C 96 days.

The acceptance criteria for stability quality control samples were %CV within 25%, and %Bias was within ±25%, at least 2/3 of the analysis batches and 50% of each concentration level should meet the above acceptance criteria.

2.7 | Data analysis

Implement the Bio-Plex 200 System for plate reading and data output. Watson LIMS V7.6.1 was used for standard curve regression (four-parameters fit) and sample concentration calculation, and Microsoft Excel 2013 was used for summarizing data and reporting. The antibody concentration in the participant's serum will be calculated by returning the sample's RFI to a four-parameter equation obtained by regressing the sample's RFI from the standard curve:

$$RFI = A + \{(B-A)/[1 + ((Conc/C)\hat{}D)]\}.$$

Where RFI = relative fluorescence intensity, A = response at infinite concentration, B = response at zero concentration, C = EC50, Conc. = concentration, and D = slope parameter usually close to 1.0.

$$\%CV = (\text{Standard Deviation}/\text{Average of measured values}) \times 100\%,$$

$$\%Bias = (\text{average of measured values} - \text{theoretical value})/\text{theoretical values} \times 100\%,$$

$$\text{Total Analytical Error} = \%CV + |\%Bias|.$$

3 | RESULTS

3.1 | Calibration curve

The seven-point standard curve for each HPV type in LIA showed good linearity over the concentration range of 4–200 u/mL, with correlation coefficients (R^2) greater than 0.999 for all types. For all HPV types, %Bias ranged from −1.1% to 1.2% and %CV ranged from 0.5% to 2.5%. The 14 standard curves are shown in Figure 2.

The inhibition rate of standard curve samples in cLIA was fitted by the same method as LIA. Various standard curves have good linearity. %Bias for all HPV types ranged from −6.0% to 14.8% and %CV ranged from 0.7% to 7.0%. The 14 standard curves are shown in Figure 3.

3.2 | Accuracy and precision

A total of six batches of validation were carried out for each method, and all data met the criteria. The intraprecision of LIA was 0.6 ~ 21.6% and the inter-precision was 3.8 ~ 10.5%. The overall

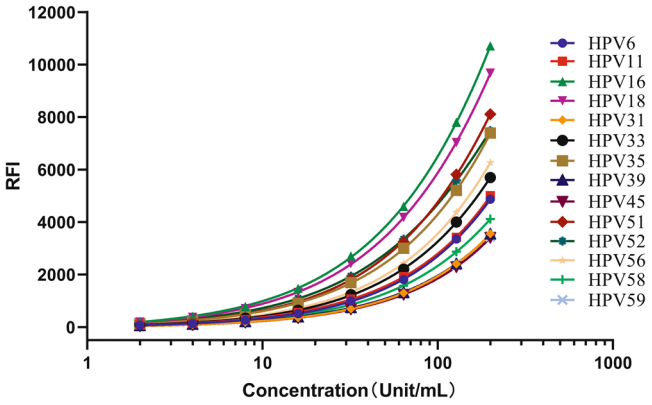

FIGURE 2 Linearity range of *anti*-HPV specific total IgG for reference standard serum. HPV, human papillomavirus.

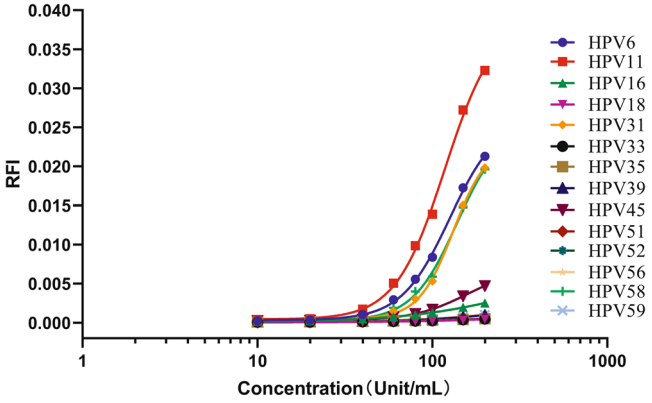

FIGURE 3 Linearity range of *anti*-HPV neutralizing antibody for reference standard serum. HPV, human papillomavirus.

analytical error range was 6.1 ~ 31.9%. The intra-precision of cLIA was 0.0 ~ 21.8% and the inter-precision was 3.3 ~ 18.1%. The overall analytical error range was 3.3 ~ 28.8%. Both LIA and cLIA methods showed good accuracy and high precision for all types.

3.3 | Selectivity

3.3.1 | Selectivity was validated for LIA and cLIA methods

For LIA, % CVs for selective samples prepared from blank matrix of normal individuals ranged from 0.0% to 16.7%, whereas %CVs ranged from 0.0 ~ 9.4% to 0.0 ~ 9.6% for hemolyzed and lipemic samples, respectively. The %Bias of blank matrix, hemolyzed samples and lipemic samples of normal subjects were −16.2 ~ 19.8%, −19.0 ~ 8.3%, and −22.3 ~ 8.3%, respectively.

For cLIA, the %CVs for blank matrix-prepared selective samples from normal individuals ranged 0.0% to 27.2%, and the %CV for hemolyzed and lipemic samples ranged from 0.0% to 12.8% and 0.0% to 29.2%, respectively. The %Bias of normal blank matrix, hemolyzed samples and lipemic samples were −26.2 ~ 30.0%, −29.1 ~ 18.4%, and −28.5 ~ 30.0%, respectively.

3.4 | Specificity

Specificity was verified for LIA and cLIA methods. The results showed that the %CV for specific samples ranged from 0.0% to 23.8% for LIA and 0.0% to 17.2% for cLIA.

3.5 | Dilutability

Samples with a theoretical concentration above the ULOQ had an RFI above the ULOQ, indicating no hook effect. Samples with a theoretical concentration below the LLOQ had an RFI below the LLOQ. Accuracy for samples ranged from −11.1% to 4.7% bias, and %CV for LIA ranged from 0.4% to 4.7%. Accuracy for samples ranged from −8.8% to 21.5% bias, and %CV for cLIA ranged from 0.5% to 7.8%.

3.6 | Stability

Stability verification results met the acceptance criteria. According to the requirements of USP 40 1106 and the European Bioanalysis Forum on the long-term stability of *anti*-drug antibody samples,[23,24] the serum samples of this project are shown to be stable for 2 years at −90 to −60°C.

The method validation summary data for the LIA and cLIA methods are shown in Tables 1 and 2.

4 | DISCUSSION

To evaluate the immunogenicity of multivalent vaccine, a high throughput and reliable assay is indispensable. Neutralizing antibodies as a major effector in preventing HPV infection[25] can be detected by PBNA, which has been widely accepted by regulatory authorities. Cervarix® used direct ELISA to complete the immunogenicity evaluation in clinical trial[26] which has the same working principle as LIA. The data show that direct ELISA have a good correlation with PBNA. The Pearson correlation coefficient of HPV16 is ≥0.70 and that of HPV18 is ≥0.82.[13] The good correlation between direct ELISA and PBNA indicated that neutralizing antibodies are mainly produced after immunization with proper HPV vaccines, accounting for a large part of type-specific total IgG. Therefore, LIA can be detected as a surrogate for PBNA for immunogenicity assessment.

In this study, two immunogenicity evaluation methods based on Luminex system and xMAP technology platform were developed, in which LIA can detect type-specific total IgG antibody levels and cLIA can quantify specific neutralizing antibodies in serum samples.

TABLE 1 LIA's assay validation performance summary.

Assay characteristic		HPV type 6	11	16	18	31	33	45	52	58	35	39	51	56	59	Design goal
Microsphere number		20	21	25	28	30	33	38	45	48	35	36	42	37	52	NA
VLP (μg/mL)		5														NA
Limit of quantitation (u/mL)								4 ~ 200								NA
QCs (u/mL)		HQC:160; MQC:40; LQC:10														NA
Precision (%CV)	Intra	0.6 ~ 21.6														≤25% (LLOQ, ULOQ ≤ 30%)
	Inter	3.8 ~ 10.5														≤25% (LLOQ, ULOQ ≤ 30%)
	Total	6.1 ~ 31.9														≤40% (LLOQ, ULOQ ≤ 50%)
Dilutability	%CV	0.4 ~ 4.7														≤25% (ULOQ ≤ 30%)
	%Bias	−11.1 ~ 4.7														±25% (ULOQ ± 30%)
Selectivity	%CV	0.0 ~ 16.7														≤25% (LLOQ, ULOQ ≤ 30%)
	%Bias	−22.3 ~ 19.8														±25% (LLOQ, ULOQ ± 30%)
Specificity	%CV	0.0 ~ 23.8														≤25%
Stability	%CV	0.0 ~ 7.0														≤25%
	%Bias	−25.0 ~ 14.3														±25%

Abbreviations: HPV, human papillomavirus; HQC, high quality control; LIA, Luminex immunological assay; LQC, low quality control; LLOQ, lower limit of quantitation; MQC, middle quality control; ULOQ, upper limit of quantitation; VLP, virus-like particle.

TABLE 2 cLIA's assay validation performance summary.

Assay characteristic		HPV type 6	11	31	45	16	18	33	52	58	35	39	51	56	59	Design goal
Microsphere number		20	21	30	38	25	28	33	45	48	35	36	42	37	52	NA
VLP (μg/mL)		5														NA
Limit of quantitation (u/mL)			20 ~ 200							10 ~ 200						NA
QCs (u/mL)		HQC:160; MQC:70; LQC:30														NA
Precision (%CV)	Intra	0.0 ~ 21.8														≤25% (LLOQ, ULOQ ≤ 30%)
	Inter	3.3 ~ 18.1														≤25% (LLOQ, ULOQ ≤ 30%)
	Total	3.3 ~ 28.8														≤40% (LLOQ, ULOQ ≤ 50%)
Dilutability	%CV	0.5 ~ 7.8														≤25% (ULOQ ≤ 30%)
	%Bias	−8.8 ~ 21.5														±25% (ULOQ ± 30%)
Selectivity	%CV	0.0 ~ 29.2														≤25% (LLOQ, ULOQ ≤ 30%)
	%Bias	−29.1 ~ 30.0														±25% (LLOQ, ULOQ ± 30%)
Specificity	%CV	0.0 ~ 17.2														≤25%
Stability	%CV	0.0 ~ 24.0														≤25%
	%Bias	−23.7 ~ 23.9														±25%

Abbreviations: cLIA, competitive Luminex immunological assay; HPV, human papillomavirus; HQC, high quality control; LQC, low quality control; LLOQ, lower limit of quantitation; MQC, middle quality control; ULOQ, upper limit of quantitation; VLP, virus-like particle.

Luminex system and xMAP platform offer high-throughput potential for analysis of up to 100 types simultaneously in parallel. To ensure the smooth progress of validation and samples detection, adequate preparations of critical assay reagents should be made. First, the preparation of VLP-MS conjugates should be standardized according to the characteristics of each type VLP to produce stable LIA and cLIA reagents. The high target signal and low cross-reactive signal of VLP-MS have a lot to do with the quality of the VLPs, and only high-quality

VLP will ensure the specificity of detection. Through standardized release verification, different batches of VLP conjugated MS that meet the standard can be used for verification and detection. Second, the preparation process and validation methods of PE-mAb conjugates were developed and standardized. PE-mAb were used for method validation and immunogenicity testing only when the target signal and cross-reactive signal both met the release criteria. In comparison with the reported MFI obtained by HPV VLP (Gardasil 9®) and PE-mAb of at a concentration of 0.1 μg/mL,[15] the specific mAbs used in this study showed an on-target signals nearly 10 times higher than that of a non-type-specific mAb at 10 μg/mL, so the nonspecific binding of PE-mAbs was <1%, which enhanced the assay performance and provided strong support for subsequent validation and detection.

In a previous study, we developed and validated a convenient and accurate method (LIA) for evaluating the immunogenicity of a 14-valent HPV recombinant vaccine based on a cynomolgus monkey serum matrix.[21] LIA and PBNA showed high correlation in immunogenicity results after immunization of the vaccine candidate, which supports the potential use of the LIA method in clinical trials. In this study, the LIA method was successfully updated and transferred to the immunogenicity evaluation of human serum. The LIA method based on human serum matrix was comprehensively validated, and the assay parameters were confirmed performing well, with high sensitivity and precision, good stability and good reproducibility.

In addition to the LIA method, the cLIA method, as an alternative method to PBNA, is widely used in clinical trials to detect human serum for immunogenicity evaluation. cLIA detects neutralizing antibodies like PBNA. In this study, a cLIA method was developed and validated to simultaneously detect quantitatively NAbs from up to 14 HPV types, exceeding the 9 types detected by a previous developed cLIA method by Merck. The method showed good specificity which was associated with specific PE conjugated neutralizing mAbs developed in house (Supplement). This method can only detect NAbs in serum that compete for the same epitopes as the PE-conjugated mAbs. In contrast to LIA, cLIA is able to evaluate antibodies with neutralizing activity but cannot detect all NAbs due to the limited epitopes of the individual PE-mAbs used. Similarly, the method was fully validated based on the human serum matrix, with good performance of various parameters, high sensitivity and precision, good stability and good reproducibility.

As expected, LIA showed better sensitivity than cLIA and required fewer critical reagents to be prepared. Robust multibatch PE-mAb production is another challenge when testing large numbers of samples spanning a long duration using the cLIA method due to variability of PE conjugation process.

LIA and cLIA are intended for immunogenicity assessment of prophylactic vaccines, while existing guidelines and White Papers do not adequately cover specific validation requirements for this type of immunogenicity assays.[22] Both LIA and cLIA are quantitative methods using the same reference standard serum. A review mentions that vaccine assay could be categorized as a subset of biomarker assay.[22] Therefore, the validation protocols of LIA and cLIA were designed and adjusted appropriately with reference to the validation strategy and parameters of quantitative analysis described in ICH Guideline M10 and the recommendations from White Papers related to vaccine and biomarker.[27–29] The immunogenicity assay validation for vaccines can be adapted in accordance with the ligand binding assays (LBA) validation strategy but do not have to comply fully with the criteria. The criteria could be flexible according to actual situations in development. LBA validation strategies are more rigorous and comprehensive than biomarker assay. In this study, LIA and cLIA passed all validation criteria, confirming that the LBA validation strategy is feasible for vaccine immunogenicity assays. To support the immunogenicity assessment of Phase III clinical trials, further optimization of assay methods will be performed, including but not limited to initial dilution and baseline/cutoff confirmation with actual human sera.

In summary, to support the clinical development of a novel VLP-based 14-valent recombinant HPV vaccine with a broader range of cervical cancer protection (above 95%) than Gardasil 9® (less than 90%), it is necessary to develop a high-throughput method for immunogenicity assessment. The method should be able to identify and quantify the levels of specific antibodies against each type of up to 14 HPV species. Therefore, we focused on the Luminex or xMAP platform to develop LIA and cLIA methods to meet the requirements. Both methods were developed and thoroughly validated based on human serum matrix. These methods show good performance on all analytical parameters. In contrast, LIA showed better sensitivity than cLIA and was not limited by the mAb development and PE-mAb conjugation process, which is also a challenge to achieve detection in a large number of HPV types and clinical samples. The validation of this study not only supports the detection of human serum samples in clinical trials, but also provides a set of validation strategies and usable standards for general vaccine immunogenicity assay. LIA and cLIA have been applied in both Phase I and II immunogenicity evaluations of the 14-valent HPV vaccine. Correlation analyzes between methods for these human samples will be performed and reported in the future.

AUTHOR CONTRIBUTIONS

Conceptualization, planning, and management: Liangzhi Xie, Yang Wang, Chunxia Luo, and Wenlin Gai. *Supervising the study conducting*: Jing Li, Xuefeng Li, and Wenlin Gai. *Method developing, critical reagents validation, and transferring*: Xiao Zhang and Dan Meng. *Critical reagents preparation*: Chaonan Zhao, Rui Wang, Lu Zhao, and Ping Hu. *Performing experiments and analysis*: Hong Li. *Supervising experiments*: Xiao Zhang, Dan Meng, Jing Li, and Weihua Gu. *Writing the manuscript*: Xiao Zhang and Dan Meng. *Reviewing and editing the manuscript*: Xuefeng Li, Yang Wang, and Xiao Zhang. All authors have read and agreed to the published version of the manuscript.

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CONFLICTS OF INTEREST STATEMENT

Xiao Zhang, Dan Meng, Xuefeng Li, Jing Li, Ping Hu, Lu Zhao, Rui Wang, Chaonan Zhao, Chunxia Luo, Wenlin Gai, Yang Wang, and Liangzhi Xie are employees of Sinocelltech Ltd. and have ownership or potential stock options in the company. Hong Li and Weihua Gu are employees of Shanghai Xihua Scientific Co., Ltd. Validation was performed at Shanghai Xihua Scientific Co., Ltd under the commitment of Sinocelltech Ltd. The remaining authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

Data supporting the findings of this study are available from the corresponding author upon reasonable request.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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