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Research Article

Development of a 2-Plex Luminex-Based Competitive Immunoassay to Quantify Neutralizing Antibodies Induced by Virus-Like Particles for Human Papillomavirus 16 and 18

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Human papillomavirus (HPV) L1 virus-like particles (VLPs) were proven an effective vaccine candidate to prevent against HPV-16 and -18 infections. In order to evaluate the potency of our produced HPV-16 and -18 L1 VLPs-based vaccine candidates, also to quantify neutralizing antibodies induced by them, a 2-plex Luminex-based competitive immunoassay was developed. Unlike the published paper, the no-biotin conjugated neutralizing mAbs spiked normal human serum (NHS) was used for standard curve preparation, while phycoerythrin (PE) was not labeled directly to neutralizing mAbs for signaling. After the coupling optimization of VLPs to microspheres and the neutralizing mAbs biotinylation, the 2-plex standard curve was prepared with good fit and high dynamic range. In addition, no cross-reactivity was also confirmed. The 2-plex Luminex-based immunoassay represents good potential not only for vaccine candidate's evaluation but also for its further clinical use.

1. Introduction

Cervical cancer is the second most frequent cancer in women in the world, comprising about 10% of all human cancers [1]. A current summary report from WHO indicates that there are about 500 thousand women diagnosed with cervical cancer, of whom about 270 thousand women die from the disease every year [2]. In China, a total of 500 million women are at risk of cervical cancer [3].

After more than 30 years of research into the infectious nature of cervical cancer, it is well established that human papillomaviruses (HPVs) are the primary cause for cervical cancer [4]. Nearly all cervical cancer cases are linked to genital infection with HPVs. Up till now, over 130 different types of HPV have been found, where HPV-16 and HPV-18 are of high risk, contribute to over 70% of all cervical cancer cases [5]. Therefore, prevention of HPV-16 and HPV-18's infection among women becomes an important issue for scientists and epidemiologists to lower the incidence of cervical cancer.

HPVs are double-stranded DNA viruses, which target the basal cells of squamous epithelia for infection [6]. The structure of its circular DNA genome are composed of 2 major oncogenes E6 and E7, 2 major structural protein genes L1 and L2 [7]. Based on these elements, different vaccines have been developed or are under development. The L1 protein of HPV expressed recombinantly in vitro can self-assemble into virus-like particles (VLPs). VLPs have HPV type-specific conformational neutralizing epitopes and thus induce type-specific neutralizing antibodies to protect against HPV infections [8]. In line with this fact, large pharmaceutical companies like Merck and GSK have developed VLP-based vaccine products and successfully put them into the market, using yeast and insect cell expression systems, respectively [1]. Both of these two products include VLPs for HPV-16 and HPV-18. In our company, an L1 VLP-based vaccine candidate aiming at HPV-16 and HPV-18 was also produced using yeast protein expression system [9, 10]. It includes VLP-16 and VLP-18 and thus should prevent against over 70% of cervical cancers. For L1 VLP-based vaccines, the efficacy is

largely dependent on the number of neutralizing antibodies induced by the conformational neutralizing epitopes existing on VLP surfaces. The more the neutralizing antibodies are induced, the more the vaccine should be effective to protect against HPV infections.

To date, different methods have been developed to quantitate the type-specific neutralizing antibodies for HPVs, such as pseudoneutralization assays [11] and cRIAs [12]. All these methods have limitations as being time consuming or less sensitive. For this reason, several studies have used a Luminex-based competitive immunoassay [13, 14] which could be used to quantify different type-specific neutralizing antibodies in a single reaction simultaneously. In the assay, different types of VLPs were bound with different kinds of microspheres, then the known, HPV type-specific, phycoerythrin-labeled neutralizing monoclonal antibodies (mAbs) were mixed with VLP-coupled microspheres and the vaccine challenged serum samples for reaction, where the PE-labeled neutralizing mAbs compete with the serum antibodies to bind to the conformational epitopes on VLPs. After that, the microspheres were put into Luminex systems for detection of the PE signal. Therefore, strong signals mean more PE-labeled mAbs binding, and thus less neutralizing antibodies existed in the serum sample. In order to quantitatively evaluate the high and low levels of neutralizing antibodies, reference standards are essential. Serum from African green monkeys hyperimmunized with HPV L1 VLPs was reported [14] to be used as reference standards successfully. An alternative is to make use of normal human serum spiked with unlabeled type-specific neutralizing monoclonal antibodies as reference standards.

In this study, a 2-plex Luminex-based competitive immunoassay was developed to evaluate the efficacy of our HPV-16 and HPV-18 L1 VLP-derived vaccine candidates. The reference standard was prepared using normal human serum spiked with various amounts of no-fluorescence labeled type-specific neutralizing mAbs rather than HPV-positive serum. The fluorescence-labeled neutralizing mAbs were not coupled directly with PE but with biotin first, and an amount of streptavidin-PE was added then for detection, which makes the assay more flexible. In developing the assay, at first, the coupling ratio of VLPs to microspheres and the coupling ratio of biotin to type-specific neutralizing mAbs were optimized, the mixed VLP-coupled microspheres were blended with the mixed biotinylated neutralizing mAbs to confirm the lack of cross-neutralizing activity, and finally a 2-plex standard curve for the competitive immunoassay was constructed for neutralizing antibody detection in serum samples.

2. Materials and Methods

2.1. Reagents and Equipment. In this study, the microspheres Bio-Plex COOH Bead 038 and Bio-Plex COOH Bead 053 were purchased from Bio-Rad Corporation, Hercules, Calif. The HPV-16 L1 VLP and HPV-18 L1 VLP were produced by yeast protein expression system in the laboratory of Shanghai Zerun Biotechnology Co., Ltd. Shanghai, China. The neutralizing monoclonal antibodies (mAbs) H16.V5 for HPV-16 and H18.J4 for HPV-18 were kindly provided by Dr. Neil Christensen, Penn State University, Penn State, USA. The normal human serum was purchased from a local hospital, Shanghai, China.

The Luminex 200 system was purchased from Luminex Corporation, and data was collected and analyzed with Luminex IS 2.3 and Prism 4. Microspheres were incubated in 96-well 1.2 μm PVDF filter microtiter plates (Millipore, Billerica, Mass) and rotated in MS 3 digital (IKA, Janke & Kunkel-Str. 10 D-79219 Staufen, Germany). The washing steps were carried out in Bio-Plex Pro II Wash Station (Bio-Rad, Hercules, Calif).

2.2. Coupling of HPV L1 VLPs to Microspheres. VLPs were coupled to microspheres according to the previously published methods with a few modifications [13, 14]. HPV-16 L1 VLP (VLP-16) and HPV-18 L1 VLP (VLP-18) were covalently conjugated to Bio-Plex COOH Bead 038 (#38 beads) and Bio-Plex COOH Bead 053 (#53 beads), respectively, as follows.

Initially carboxylated beads (1.25×10^7 beads/mL) were fully suspended by vortexing and sonication, and then about 500 μL beads were transferred to a 1.5 mL Protein LoBind Tube (Eppendorf) for coupling. After centrifuging the tube at 14000 g for 4 min, the supernatant was removed. Then the beads were washed once with 500 μL 0.1 M NaH_2PO_4 (pH 6.2) by vortexing and sonication, followed by an incubation with 50 μL 50 mg/mL sulfo-NHS (Pierce, Rockford, IL), 70 μL 0.4 M 1-ethyl-3-[3-dimethylaminopropyl]carbodiimide hydrochloride (EDC) (GE, Shanghai, China), and 380 μL PBS (pH 7.4) for activation, where amine-reactive esters of carboxylate groups were formed for labeling. The activation lasted in darkness for 30 min, at 800–1000 rpm, RT. After that, the beads were washed twice with 50 mM MES buffer (Sigma, pH 6.0) and pelleted in MES buffer before counting using a hemocytometer.

The HPV L1 VLPs were transferred to 50 mM MES buffer (pH 6.0) by ultrafiltration, and then its concentration was determined by BCA (BCA protein assay kit, Pierce, Rockford, Ill) before use.

In coupling, 1×10^5 activated beads and buffer with transferred VLPs were mixed in MES buffer to a final volume of 100 μL–200 μL. The mixture was rotated in darkness, at 800–1000 rpm, RT, overnight. Consequently, the beads were washed twice with PBS plus 0.05% Tween-20 (pH 7.4), followed by blocking in histidine buffer (20 mM histidine (Sigma), 0.5 M NaCl, 1% BSA, pH 6.2) for 30 min in darkness, RT, at 800–1000 rpm. After washing the beads twice with histidine buffer, they were counted by hemocytometer and diluted to 1×10^6 beads/mL in histidine buffer for preservation in darkness, at 4°C.

2.3. Neutralizing mAbs Biotinylation. The HPV-16 and HPV-18 neutralizing mAbs H16.V5 and H18.J4 were biotinylated using Micro Sulfo-NHS-LC-Biotinylation Kit (Pierce). The procedure followed the manufacturer's instructions. In brief, neutralizing monoclonal mAbs were transferred to PBS (pH 7.2–7.4) and subjected to BCA (BCA protein assay

kit, Pierce) to determine concentration before biotinylation. An amount of active Sulfo-NHS-LC-Biotin for different mole ratios of biotin and mAb was then added, and the mixture was rotated at 800–1000 rpm in darkness, RT, for 45 min. Finally, the biotinylated beads were put at −20°C immediately to stop the reaction and stored before use.

2.4. Confirming the Neutralizing mAbs Biotinylation. The biotinylation of H16.V5 and H18.J4 was confirmed to determine the optimal mole ratio of biotin and mAbs for reaction. The process was described as follows. In a prewetted 96-well microtiter filter plate, 5000 VLP-16 conjugated #38 beads (25 μL, 2×10^5 beads/mL, Coupling ratio = 40 μg protein per million beads) or VLP-18 conjugated #53 beads (25 μL, 2×10^5 beads/mL, Coupling ratio = 40 μg protein per million beads) were mixed with 25 μL of diluted biotinylated H16.V5 (Biotin/H16.V5 mole ratio = 20/1, 40/1, 80/1, 160/1, 320/1, 640/1, 1280/1) or biotinylated H18.J4 (Biotin/H16.V5 mole ratio = 20/1, 40/1, 80/1, 160/1, 320/1, 640/1, 1280/1) and 50 μL PBS-BSA to a final volume of 100 μL, where the biotinylated mAbs were 20-fold diluted, followed by a 2-fold serious dilution in a total of 11 different dilutions. Then the microtiter plate was rotated at 800–1000 rpm, in darkness, for 3 h, followed by 3 times of wash in Wash Station. After that, 50 μL 2.5 μg/mL streptavidin-PE was added for incubation at 800–1000 rpm, in darkness, for 20 min. The beads were then washed 3 times and resuspended in 125 μL PBS-BSA for reading in Luminex 200 system (Luminex Corporation, Austin, Tex).

2.5. Confirming the Coupling of HPV L1 VLPs to Microspheres. After the coupling reaction of HPV L1 VLPs to microspheres has been completed, the coupling efficiency was confirmed. In brief, 5000 VLP-16 conjugated #38 beads (25 μL, 2×10^5 beads/mL) or VLP-18 conjugated #53 beads (25 μL, 2×10^5 beads/mL) were mixed with 25 μL biotinylated H16.V5 (Biotin/H16.V5 mole ratio = 100/1) or biotinylated H18.J4 (Biotin/H18.J4 mole ratio = 100/1) and 50 μL PBS-BSA buffer (PBS, 1% BSA, pH 7.4) to a final volume of 100 μL, for incubation in a 96-well prewetted filter plate, in darkness, at 800–1000 rpm for 3 h, where the biotinylated mAb was 1 : 50, 1 : 100, 1 : 1000 diluted in PBS-BSA buffer (PBS, 1% BSA, pH 7.4). Then the beads were washed 3 times in Bio-Plex Pro II Wash Station (Bio-Rad) using PBS-BSA buffer. After that, 50 μL 2.5 μg/mL streptavidin-PE was added, followed by incubation in darkness, at 800–1000 rpm for 20 min. After washing the beads again for 3 times with the Wash Station, 125 μL PBS-BSA buffer was added for reading in Luminex 200 system (Luminex Corporation). All assays were made in triplicates.

2.6. 2-Plex Standard Curve Preparation for Competitive Immunoassay. In this study, the normal human serum spiked with different amounts of the no-biotin labeled neutralizing mAbs was used as the standard. It was prepared as follows: initially the normal human serum (NHS) was blended with certain amounts of unlabeled H16.V5 and H18.J4 to a final concentration of 20 μg/mL. Then, the mixture was 2-fold diluted with NHS to make H16.V5 and H18.J4 in a series of 15 different concentrations. For each 50 μL diluted sample, 5000 VLP-16 conjugated #38 beads (Coupling ratio = 40 μg protein per million beads), 5000 VLP-18 conjugated #53 beads (Coupling ratio = 40 μg protein per million beads), 500-fold diluted (final dilution) biotinylated H16.V5 (Biotin/H16.V5 mole ratio = 320/1), and 200-fold diluted (final dilution) biotinylated H18.J4 (Biotin/H18.J4 mole ratio = 640/1) were added to a final volume of 100 μL, followed by an incubation in 96-well prewetted filter plate, in darkness, at 800–1000 rpm for 3 h. After washing in triplicate by Wash Station using PBS-BSA buffer, the beads were added with 50 μL 2.5 μg/mL streptavidin-PE and rotated at 800–1000 rpm, in darkness, for 20 min for staining. Finally the beads were washed again in triplicates and resuspended in 125 μL PBS-BSA buffer before reading in Luminex 200 system (Luminex Corporation). All assays were conducted in duplicates.

2.7. Cross-Reactivity. In brief, 5000 VLP-16 conjugated #38 beads (Coupling ratio = 40 μg protein per million beads) and 5000 VLP-18 conjugated #53 beads (Coupling ratio = 40 μg protein per million beads) were added with 40-fold diluted (final dilution) biotinylated H16.V5 (Biotin/H16.V5 mole ratio = 320/1) and 200-fold diluted (final dilution) biotinylated H18.J4 (Biotin/H18.J4 mole ratio = 320/1) or both. All assays were conducted in duplicate.

3. Results

3.1. Determine the Optimal Coupling Ratio of HPV L1 VLPs to Microspheres. Different ratios (μg/million beads) of HPV-16 L1 VLP or HPV-18 L1 VLP and #38 or #53 microspheres were used for coupling, respectively. The VLP coupled microspheres were then reacted with the same amount of related neutralizing mAbs. The coupling ratio at which the VLP-conjugated beads can generate the strongest signal was then selected as the optimal. Different dilutions of neutralizing mAbs led to the same results. The coupling reaction for VLP-16 and #38 beads was saturated when coupling ratio was bigger than 40 μg protein per million beads (Figure 1(a)), while for VLP-18 the optimal coupling ratio was 40 μg protein per million beads (Figure 1(b)). The results may be due to their specific dimensional structures and different neutralizing epitopes. Another concern for choosing 40 μg protein per million beads was to find a robust way for coupling; more VLPs used could ascertain the same quality of coupled beads at each time. It would be important in QC and clinical trial.

3.2. Determine the Optimal Coupling Ratio for Neutralizing mAbs Biotinylation. For biotinylation of neutralizing mAbs H16.V5 and H18.J4, different coupling mole ratios of amine reactive biotin and mAb were tried, and the biotinylated mAbs were then mixed with VLP-conjugated microspheres for reaction. The optimal coupling mole ratio was the one at which the reaction could generate the strongest signal. For both H16.V5 and H18.J4, the optimal coupling ratios of biotin to mAbs were 320/1 or higher (Figures 2(a) and 2(b)),

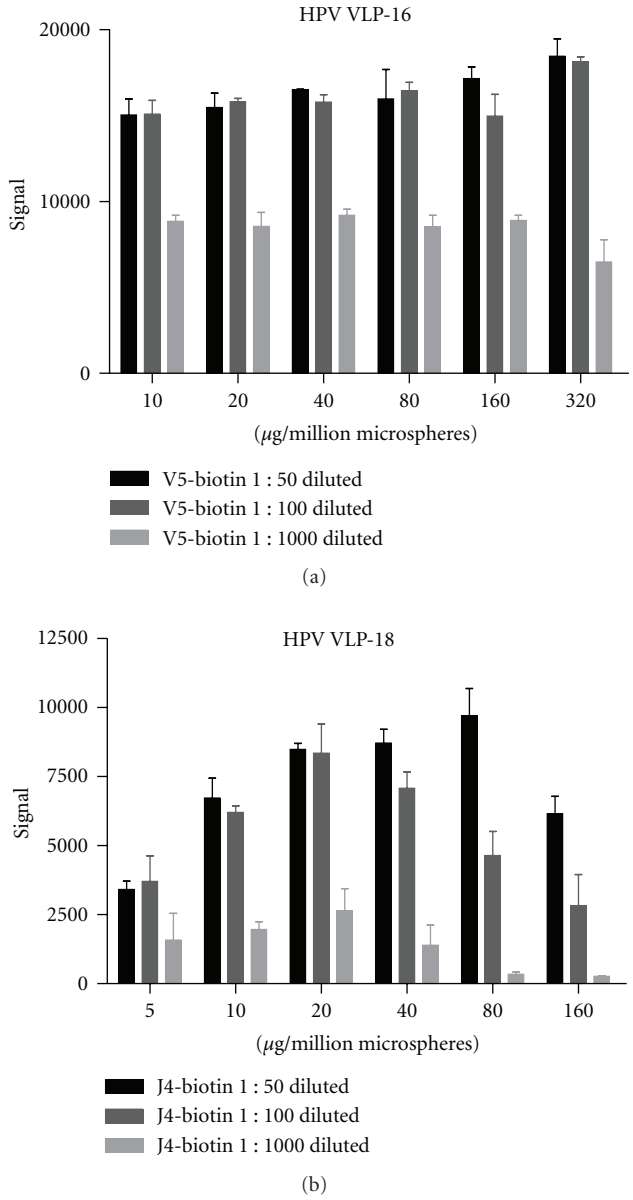

Figure 1: Coupling ratio optimization for HPV L1 VLPs and microspheres. (a) HPV-16 L1 VLP and #38 microspheres. (b) HPV-18 L1 VLP and #53 microspheres.

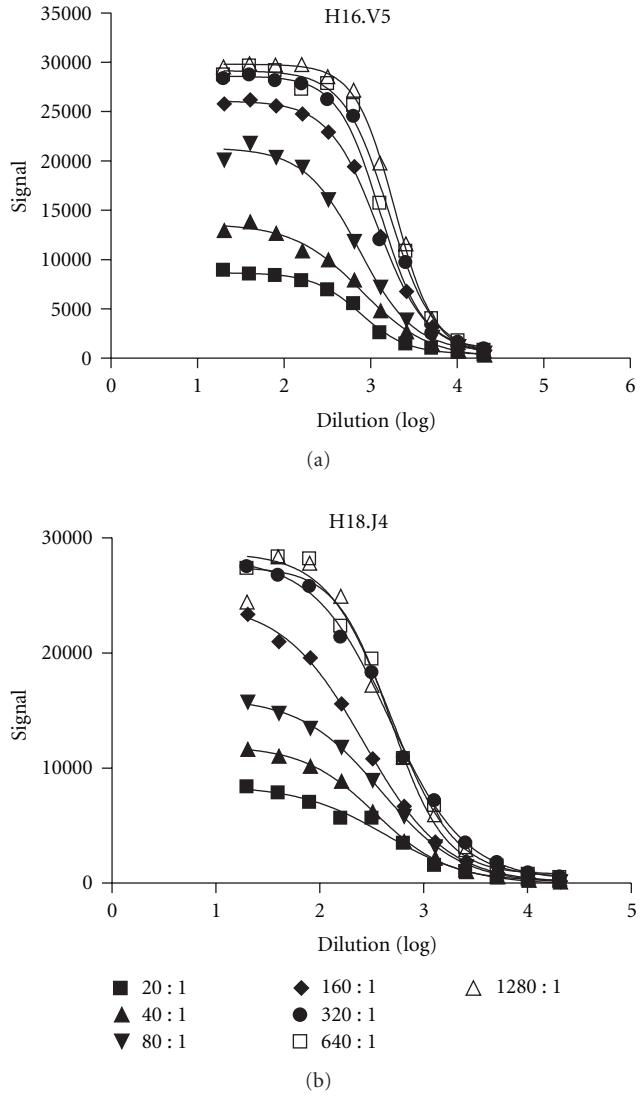

Figure 2: Coupling ratio optimization for neutralizing mAbs biotinylation. (a) H16.V5 biotinylation. (b) H18.J4 biotinylation.

where the biotinylation reaction was saturated at the current detection system. When changing our system, for example, the added amount and concentration of streptavidin-PE, the optimal coupling ratio for biotinylation may be higher, because there may be not enough streptavidin-PE for staining.

3.3. No Cross-Reactivity. Before mixing the 2 VLP-conjugated microspheres together for immunoassay, studies were carried out to make sure that no cross-reactivity existed between the 2 analytes. Just as predicted, with our produced HPV vaccine candidates, biotinylated H16.V5 could only bound to VLP-16 coupled microspheres for signaling while biotinylated H18.J4 only bound to VLP-18 coupled microspheres (Figure 3). The results ascertained the establishment of the 2-plex immunoassay with high sensitivity and specificity. In this section, the saturated VLP coupled beads plus the saturated biotin coupled mAb just generate no cross-reactivity.

3.4. 2-Plex Standard Curve Preparation for the Luminex-Based Competitive Immunoassay. The optimized conditions were adopted for standard curve (STD) preparation. The coupling ratios for VLPs to microspheres were both 40 μg protein per million beads. The biotinylation ratios for biotin to neutralizing mAbs were 320/1 or higher. Not surprisingly, standard curve preparations (Figure 4) for the 2-plex Luminex-based competitive immunoassay were established successfully with good fit ($R^2 > 0.99$) for both analytes and wide dynamic range (9.8 ng/mL to 5.0 μg/mL for VLP-16 neutralizing antibodies, while 39.0 ng/mL to 5.0 μg/mL for

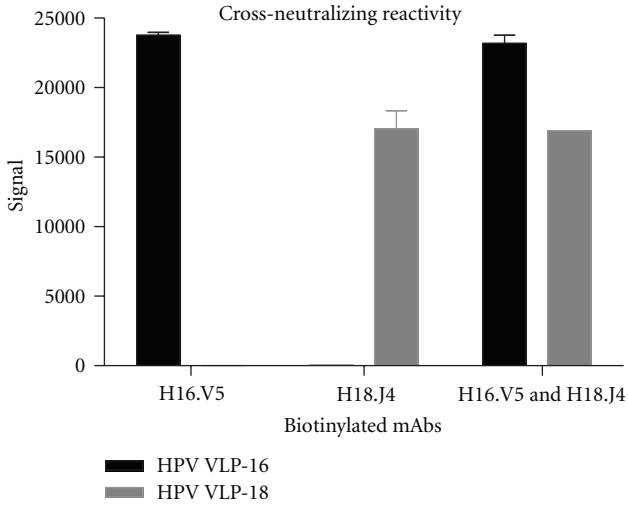

Figure 3: Cross-neutralizing reactivity investigation.

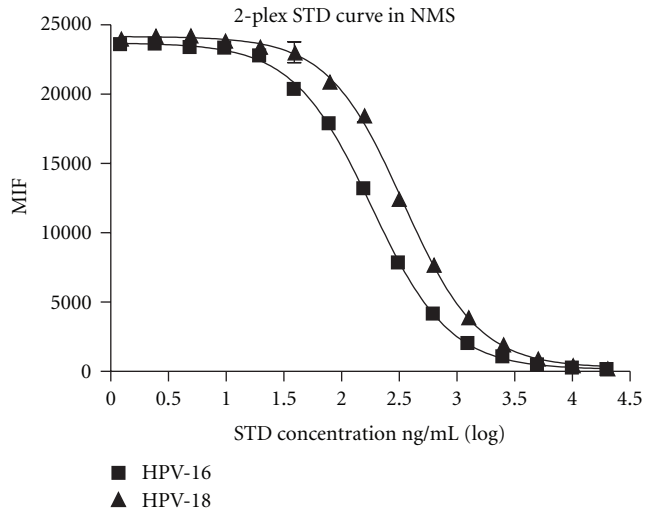

Figure 4: 2-plex standard curve for the Luminex-based immunoassay.

VLP-18 neutralizing antibodies), where the no-biotinylated mAbs (standards) and biotinylated mAbs competitively bind to HPV VLP coupled microspheres.

4. Discussion

Luminex xMAP system is a technology combined with flow cytometry, microspheres, and lasers together, which allows the quantitation of up to 100 different analytes simultaneously using quite small sample volumes in a single reaction [15]. It has been applied to HINI detection [16], kidney biomarkers detection [17], human leukocyte antigen genotyping [18], cytokine detection and quantitation [19], and so forth. with high sensitivity and specificity.

Cervical cancer, as the second prevalent cancer in women in the world, was caused by HPV infections. Some studies have revealed that HPV L1 VLP-based vaccine could effectively induce type-specific neutralizing antibodies to HPVs and thus protect against their invasions [1]. In order to make the vaccine more powerful, it was often designed to target more than one type of HPVs, for instance, Merck's Gardasil aimed at HPV-6, -11, -16, and -18 simultaneously. As a sensitive, economic and high-throughput method, Luminex system was reported [13, 14] to evaluate the efficacy of the HPV L1 VLP-based vaccine products. Compared to the traditional ELISA- or FACS-based method, Luminex has many advantages. It could be used to quantitate type-specific neutralizing antibodies induced by various HPVs simultaneously and thus was more practical for clinical use, where large number of serum samples should be analysed in a short time. Here, in order to establish an assay for efficacy determination of our HPV-16, -18 L1 VLPs based vaccine candidate, also for further clinical use, a 2-plex Luminex-based competitive immunoassay was also developed. Although the reported method represents an important reference for this study, some modifications were tried to make the assay more flexible in the practical use.

When developing the immunoassay, first of all, the amount of VLP needed for coupling with microsphere should be determined to generate constant and strong signals. For the 2-plex method, 2 different beads were prepared for HPV-16 L1 VLP and HPV-18 L2 VLP, respectively. Interestingly, the optimal coupling ratio for VLP-18 was 40 μg/million beads, while for VLP-16 the coupling would be saturated if the ratio was bigger than 40 μg/million beads, which may be due to the different neutralizing epitopes existed in VLP-16 and VLP-18. On the other hand, in this step, it was also found that low-binding tube was very essential for coupling of high quality (data not shown), while the conventional tube may absorb microspheres during the reaction and thus lower the recovery.

The amount of biotin needed for biotinylation of neutralizing mAbs should also be optimized. In the reported method, the signal dye PE was bound directly to mAbs, which we thought was inflexible for the assay's practical use. Sometimes, it is difficult to obtain a stable PE labeled mAb or other molecules when several new analytes are to be added to this multiplex assay. In contrast, biotin labeling technology is highly developed, and it is simple to obtain stable biotinylated mAb. In fact, biotinylated mAb plus streptavidin-PE was commonly used for signaling in most ELISA, Luminex, or other immunoassay-based systems. In this study, the optimal ratios for biotinylation of H16.V5 and H18.J4 were determined, respectively.

Just as predicted, with our produced HPV-16 and HPV-18 L1 VLPs based vaccine candidate, no cross-activity was found between H16.V5 and H18.J4, which were well-characterized, strong type-specific HPV-16 and HPV-18 neutralizers. After that, the 2-plex standard curve (STD) of the competitive immunoassay for quantifying antibodies neutralizing HPV-16 and HPV-18 simultaneously was then tried to be constructed. Unlike the published paper from Merck, normal human serum spiked with no biotin labeled neutralizing mAbs was used for standards, instead of VLP

challenged monkey serum. The major reason for that is the difficulty in preparing enough well-characterized and standardized serum for the assay. In this study, the 2-plex STD for HPV-16 and HPV-18 was well established with high sensitivity and wide dynamic range. Later the assay was tried to measure the induced neutralizing antibodies in VLP challenged mice serum. It could be distinguished with different antibody levels for HPV-16 and HPV-18 simultaneously, of samples derived from mice challenged with our developed VLP vaccine candidate of different doses (data not shown). Therefore, the 2-plex Luminex-based assay provided a fast, sensitive, and effective approach to evaluate the efficacy of our HPV L1 VLP-based vaccine candidate.

At present, the dilution buffer used for assay was normal human serum for clinical sample use. When applying the assay to monkey or mouse serum samples, the dilution buffer should better use normal monkey serum or normal mouse serum accordingly because it is commonly used as the dilution matrix or blank. The published paper suggests the use of antibody-depleted human serum (ADHS) as dilution buffer because of its reliable and stable resources, which we thought could also be tried in our developed systems in the future. In addition, "the unit" used for characterizing the level of neutralizing antibodies in serum samples should be defined. In doing this, large number of serum samples with neutralizing antibodies of low, medium, and high levels should be applied to the same STD curve, to further validate the assay.

5. Conclusions

A 2-plex Luminex-based competitive immunoassay was developed to evaluate the efficacy of our HPV L1 VLP-based vaccine candidate in inducing neutralizing antibodies against HPV-16 and HPV-18. Some small but essential modifications have been made, compared to the reported publications. The assay was convenient, sensitive, effective, and requiring only small amount of samples. Recently, additional studies from our laboratory are underway to further validate the assay and also determine the method limitation with plenty of serum samples. The correlations of the assay to other characterization method for HPV L1 VLP-based vaccines like pseudoneutralization assay [11] and IVRP [20] were also investigated. Moving forward, as researches on the assay become more comprehensive, the 2-plex Luminex assay will be routinely applied for evaluating and quantifying neutralizing antibodies in clinical use.

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