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製備胜肽生物標誌物及其抗體以開發偵測口腔癌患者唾液中之人類乳突病毒相關生物標記物的生物感測器/生物晶片為基礎之方法

Preparation of Peptide Biomarkers and Their Antibodies for the Development of Biosensor-/ Biochip-based Methods for Detecting HPV-related Biomarkers in Saliva of Oral Cancer Patients

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摘要

近三十年來癌症高居國人死因之首，目前口腔癌為台灣十大癌症死因中的第五順位。而罹患口腔癌之危險因子中，人類乳突病毒 (HPV) 為近年研究的新目標，由 HPV 引起的口腔癌之預後情況及治療方法與傳統危險因子所引起的口腔癌有所不同，因此本研究欲開發非侵入式且方便的唾液篩檢工具，篩選出受到 HPV 感染高風險的口腔癌患者，並立即制定治療方針，以降低醫療成本。本研究首先選擇唾液中 HPV 16 型 E7 蛋白作為模板，設計出一胜肽片段 (命名為 HP-3 胜肽)，並應用固相胜肽合成法 (SPPS) 合成後，使用逆相高效能液相層析儀 (RP-HPLC)純化與分析，並以基質輔助雷射脫附游離飛行時間質譜儀 (MALDI-TOF MS) 鑑定其分子量;接著將 HP-3 胜肽作為抗原注射雞隻以免疫製備其雞蛋黃多株抗體，以西方墨點法確認胜肽與抗體間之專一性; 最後以表面膜漿共振生物感測器鑑定胜肽與抗體之效價，並進行檢體中抗原抗體交互作用力分析以偵測口腔癌患者唾液中相關的蛋白質或抗體。研究結果證實我們開發出非侵入性、生物感測器為基礎的方法，可應用於檢測口腔癌患者唾液中 HPV 相關的生物標誌，且對篩選出 HPV 引起之口腔癌患者具有相當大的潛力。

關鍵字：

口腔癌、人類乳突病毒、固相胜肽合成法、生物標記物、表面電漿共振、生物感測器

Abstract

Over the past three decades, cancer has become the leading causes of death in Taiwan, and oral cancer ranks fifth in Taiwan's top ten cancers. Among the risk factors of oral cancer, human papillomavirus (HPV) is a new target for recent research, the prognosis and treatment methods for HPV related oral cancer are different from those for the oral cancer caused by traditional risk factors. Therefore, this study is going to prepare the peptide biomarkers and their antibodies for the development of biosensor-/ biochip-based methods for evaluating the risk of patients with oral cancer who are at the high risk of HPV infection, and immediately prescribe a course of treatment to reduce medical costs. In this project, firstly, we designed a peptide fragment of HPV type 16 E7 protein (named HP-3), and synthesized it by solid phase peptide synthesis, followed by purification and analysis using RP-HPLC and characterization by MALDI-TOF MS. Secondly, HP-3 was used as an antigen for preparing polyclonal antibodies in chicken. The titer and specificity of anti-HP-3 antibodies were determined by Western Blot, kinetic and affinity analysis were determined by surface plasma resonance (SPR)-biosensor. Finally, SPR-based biosensor methods were developed for detecting HPV-related proteins or antibodies in saliva of oral cancer patient. Results demonstrate that we developed a non-invasive and convenient method for detecting HPV-related biomarkers in saliva collected from patients with oral cancer, and the peptide biomarker and SPR-methods have considerable potential for screening oral cancer patients with HPV infection.

Keywords:

Oral Cancer, Human Papillomavirus, Solid Phase Peptide Synthesis, Biomarkers, Surface Plasmon Resonance, Biosensor.

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Chapter 1. Introduction

According to the statistics analysis of the cause of death in Taiwan, reported by Ministry of Health and Welfare (MOHW) in 2016, cancers have been the leading cause of death for thirty-five years, and oral cancer is the fifth most common cause of cancer-related death.[1] Thus, it is important to prevent cancers and to identify cancer patients at the early stage. More than 90% of oral cavity cancer is oral squamous cell carcinoma (OSCC), which is a high impact disease in the oral cavity.[2] It has been known that tobacco smoking, alcohol abuse, and betel quid chewing play a key role in the etiology of OSCC.[3] The usual treatments of oral cancer are surgery, chemotherapy, radiotherapy, or combined therapy. Five-year survival rates for oral cavity cancer is around 50% in most countries.[4]

Gillison, *et al* have shown that OSCC is associated with human papillomavirus (HPV).[5] HPV are small double-stranded and non-enveloped DNA viruses with a genome of 8 kb, contained over 150 distinct genotypes (**Fig. 1**). Proliferating cells contain the viral genome in a latent form. Large scale of viral DNA replication, translation and functional activity of late viral proteins, and viral particle assembly are restricted to differentiating layers of skin and mucosa. HPV infected epithelial cells cause the production of warts. The genotypes of HPV can be classified to the high- and low-risk genotypes. The high-risk viruses such as HPV 16 and HPV 18 are related to about 70% of invasive cervical cancer cases.[6-8] HPV encode two different viral oncoproteins, E6 and E7, which played important roles in the cell cycle. E6 and E7 stimulated cell proliferation by activating cyclins E and A, and then interacted with cellular protein p53 and pRB, respectively.[7] Both of p53 and pRB are tumor suppressor genes and regulate cell-cycle checkpoints at the G1 phase. E6 binds to p53 for inactivating, degrading p53, and preventing the transmission of apoptotic signals. E7 combines with pRB to inhibit the activity of various cell transcription factors such as E2F, and to repress the transcription of genes required for the S phase of the cell cycle (**Fig. 2**).[9-11]

HPV E7 proteins are small, acidic polypeptides composed of approximately 100 amino acid residues. The amino terminus of E7 contains a region of sequence which is similar to a portion of the conserved region (CR) 1, the entire CR2 in the adenovirus (Ad) E1A, and related sequences in simian vacuolating virus 40 (SV40) large tumor antigen. A conserved Leu-X-Cys-X-Glu motif in the CR2 homology domain is necessary and sufficient for the association of E7 protein with the pRB. The conserved carboxyl terminal zinc-binding site is composed of two

Cys-X-X-Cys motifs separated by 29 amino acid residues.[12-13] HPV-16 E7 is 98 amino acid-long, with CR1 and CR2 located in the N-terminal half and CR3 is in the C-terminal part of the protein, which contains two CXXC zinc-binding motifs separated by 29 amino acids (**Fig. 3**).[14]

Some studies have shown that some patient with HPV-positive cancer tended to have had many oral-sex partners. Patients with HPV-positive cancer have better outcomes than cancer caused by traditional risk factors.[5] Therefore, it is necessary to develop a method for identifying oral cancer at early stage. Current detection methods for identification of cancer-related targets are PCR-based method and electrochemical sensors.[15] In order to improve the efficiency of methods for diagnosis and screening of oral cancer, our goal is to prepare peptide biomarkers of oral cancer and their antibodies for further development of biosensor/biochip based methods for screening and identifying subjects with high risk of oral cancer.

Surface plasmon resonance (SPR) based techniques have been widely used in recent years to study interactions between different biomolecules, such as antibody–antigen interactions, and to generate data which is equivalent to or in some cases superior to that obtained using ELISA. Though SPR-biosenor method provides some information similar to those obtained using ELISA, it has the advantage of real-time analysis and label-free of any binding partners.[16] In the classical SPR approach, a biomolecule (the ligand) is immobilized onto a sensor surface and the interacting (or binding) bio-particles (the analyte) becomes available from an analyzed probe. Interactions between the ligand and the analyte occur at sensor's interface in flow cell system under continues flow. The binding of immobilized and floating biomolecules lead to the accumulation of biomolecules onto the sensor's surface and, thus, results in the increase of the refractive index near to the sensor surface. Changes of the refractive index lead to the changes in surface plasmon waves (or surface plasmon polarations, SPP). Changes of the refractive index can be measured in real time and thus, provide information about the binding efficiency of biomolecules without any time-delay (**Fig. 4**).[17]

In this study, to develop efficient methods for diagnosis and screening of oral cancer, a peptide biomarker of oral cancer and their antibodies are prepared, and the biosensor-based methods are developed for screening and identifying subjects with high risk of oral cancer with or without the HPV infection.

Chapter 2. Materials and Methods

2.1 Design of peptide biomarkers

In order to develop a non-invasive method for oral cancer screening, we selected human papillomavirus type 16 E7 as our target on the basis of the study published by Kim *et al.* in 2017. The epitopes of HPV-16 E7 protein were identified by using enzyme-linked immunospot assay (ELISpot).[18] We used HPV-16 E7 protein as the template for the design of a peptide fragment of , E7 (named as HP-3), which is a 20-mer peptide, consist of the amino acid residues from position-46 to position-65 in E7 protein. Characterized

2.2 Peptide Preparation

The reagents, instruments, and manufacturers used by SPPS are summarized in **Table 1**.

The synthesis of HP-3 peptide was carried out manually in our laboratory according to standard FmocChemistry.[19-22] Briefly, the solid support, AM resin (0.125 mmole), was packed into an empty, disposable PD-10 column for the subsequent deprotection and coupling reactions. Firstly, DCM was added into the PD-10 column and mixed with the AM resin for 10 minutes (min) to swell the resin, then DCM was removed by suction. The resin was washed with DMF for three times. Secondly, the on-resin Fmoc protecting group was removed by adding 30 % piperidine/DMF into the column for 15 min-mixing, followed by washing with DMF for three times, repeated this procedure for three times to ensure the complete removal of Fmoc group. The N -Fmoc, sidechain-protected amino acid was pre-activated by mixing with the activating agent, HOBt/HBTU/DIEA (1:1:2, molar ratio), in a vial for 5 min. Next, added the activated amino acid into the column, mixed until the completion of the coupling reaction (checked by the ninhydrin test, it took about 2 hours (hr) for completion). Repeat the cycle of Fmoc-deprotection and coupling reactions until designed peptide-bound resin was produced.

The crude peptides were deprotected and cleaved from solid support with 82.5 % TFA cleavage mixture for 4 hr. The cleaved crude peptide was filtered into a Falcon tube in the filter system. Most of the TFA in the crude peptide mixture was removed by evaporation with a stream of N2 gas. After adding cold ether to the mixture, we can obtain the crude peptide

precipitate. The crude peptide precipitate was dissolved in deionized water and acetonitrile, and the peptide solution was frozen at -20℃, then lyophilized in a freeze dryer. Finally, we can obtain the crude peptide powder after lyophilization.

The reagents, instruments, and manufacturers used by RP-HPLC and MALDI-TOF-MS (matrix-assisted laser desorption/ionization time-of-flight mass spectrometry) are summarized in **Table 2**. The lyophilized crude peptide was dissolved in deionized water, then, filtered through a 0.22 μm syringe filter. The filtrate was collected for RP-HPLC purification. The column for RP-HPLC purification was a semi-preparative C18 reversed-phase column. The mobile phase for elution was the mixture of deionized water and acetonitrile, mixed in different ratios using the programmed gradient. The wavelength for detection was set at 225 nm, and the flow rate for elution was 4 mL/min. The target peptide and side products were collected and lyophilized. The major peptide products were characterized by MALDI-TOF-MS.

2.3 Preparation of chicken anti-peptide biomarker antibodies

The reagents, instruments, and manufacturers used by preparation of chicken anti-peptide biomarker antibodies are summarized in **Table 3**. HP-3 peptide was used as an antigen for preparation of polyclonal antibodies in chicken egg yolk.

Immunization of chickens with HP-3 to prepare anti-HP-3 polyclonal antibodies was operated according to the Institutional Animal Care and Use Committee Guidebook published by the Office of Laboratory Animal Welfare.[23] This experiment was performed by the graduate students of Professor Borrung Ou at the Department of Animal Science and Biotechnology in Tunghai University, Taichung, Taiwan.

Hens were kept in individual cages with food and fresh water. They were immunized subcutaneously at the leg region with HP-3 peptide mixed with complete Freund's adjuvant. For immunization, 300 μL of HP-3 peptide were emulsified with an equal volume of complete Freund's adjuvant. The hens were injected with the HP-3 peptide two and four weeks following the initial immunization. Eggs were collected and stored at 4ºC.

Based on the method that Dr. Poison published in 1990 in the Journal of Immunological Investgation, chloroform - polyethylene glycol method,[24] 15 mL of deionized water was added into centrifuge tubes, mixed well with yolk, then the same volume of chloroform was added and the mixture was shaked twice until it turned into a thick emulsion. The tubes were spun at 4,000 rpm for 5 minutes. Three separated phases were observed, from the lower regions of the tubes were an orange coloured solution of lecithin in chloroform, a semi-solid emulsion of the

yolk substances in chloroform and a watery phase of chicken serum proteins. Draw out the watery phase, the polyclonal antibody was frozen and stored at -20℃ or lyophilized and stored at dry box.

2.4 Determination of the identity of proteins by using Western Blot

The reagents, instruments, and manufacturers used by western blot are summarized in **Table 4**.

Western blot was performed using the standard immunoblotting protocols.[25] The recombinant E7 protein (30μg/mL) was electrophoresed and transferred onto PVDF membranes. Membranes were blocked with 5% (w/v) non-fat dry milk in PBST buffer overnight. Membranes were washed with PBST, then incubated overnight with chicken serum which is diluted in PBST buffer at a dilution of 1:1,000. After washing with PBST, membranes were incubated with HRP-conjugated goat-anti-chicken secondary antibody at a dilution of 1:10,000 for 1 hr at room temperature. Followed by washing with PBS, the membranes were reacted with TMB for 10 min.

2.5 Sample collection

In total, 80 participants, including 46 OSCC patients and 34 control subjects, were recruited for this study. All of the subjects involved in this study signed the institutional review board–approved consent form. Samples were collected and provided from The Division of Oral & Maxillofacial Surgery at the Oral Surgery Department in Taichung Veterans General Hospital. In order to maintain the balance of oral endocrine, the donors were asked to abstain from eating, drinking, smoking, or using oral hygiene products for at least 1 h before the collection of their saliva. Once the samples were collected, they were centrifuged at 3,000 rpm for 5 min at 4°C to remove debris and cells. The supernatant was collected and the protease inhibitor (1 tablet/10 mL) was added immediately to ensure the preservation of the protein integrity. The samples were immediately aliquoted into smaller volumes and stored at −80°C.

2.6 Development of biosensor/biochip based methods for detecting peptide biomarkers

The reagents, instruments, and manufacturers used by SPR are summarized in the **Table 5**.
SPR-Biosensor experiments were performed using the Biacore X100 instrument[26] at the Biomedical Platform and Incubation Services Division in the National Applied Research

Laboratories Instrument Technology Research Center in Taiwan, performed with the guidance by Dr. Yijun Hu. All experiments were conducted at 25 °C on the Biacore X100 instrument, HBS-EP was used as a running buffer, and SPR data were evaluated with BIA evaluation. Experimental procedures are listed as the following:

A. pH scouting in wizard

To determinate the optimal pH for the ligand, performed pH scouting before ligand immobilization. Used HBS-EP as running buffer. Diluted ligand to a final concentration of 30 μg/mL in 10 mM acetate buffer of different pH (pH5.5、pH5.0、pH4.5、pH4.0).A regeneration step using 50 mM NaOH is performed after each pH scouting step.

B. Immobilization

With Manual Run, HBS-EP was used as running buffer at a flow rate of 10 μL/min throughout the immobilization procedure. Flow cell 1 of CM5 sensor chip as a blank. First, EDC and NHS were mixed 1:1 and injected onto each flow cell 1 to activate the surface of the CM5 sensor chip. Injected 1M ethanolamine–HCl to inactivate the sensor chip surface.

The protein and antibody samples were immobilized on the surface of a CM5 sensor chip by amine-coupling method according to the wizards.

C. Kinetic/Affinity studies

Wizards from Biacore control software was used for obtaining all data by monitoring the change in the refractive index as a function of time at a flow rate of 30 μL/min.

Injection Sequence

Detection: Flow cell: 1, 2

Chip: Chip type: CM5

Injections in analysis cycle: Sample: 1；Regeneration: 1

System Preparation

Prime: Prime before run

Startup: Run startup cycles；Solution: Buffer；Number of cycles: 1

Injection Parameters

First sample: Contact time: 180 (s)；Dissociation time: 600 (s)

First regeneration: Solution: 3M NaCl；Contact time: 30 (s)；Stabilization period: 60 (s)。

D. Binding Analysis

Injection Sequence

Detection: Flow cell: 1, 2

Chip: Chip type: CM5

Injections in analysis cycle: Sample: 1；Regeneration: 1

System Preparation

Prime: Prime before run

Startup: Run startup cycles；Solution: Buffer；Number of cycles: 1

Injection Parameters

First sample: Contact time: 180 (s)；Dissociation time: 300 (s)

First regeneration: Solution: 3M NaCl；Contact time: 30 (s)；Stabilization period: 60 (s)。

2.7 Statistical Analysis

To determine whether the saliva sample is positive for HPV, the SPR signals obtained from the saliva samples of non-orbital cancer subjects were done quartile, also known as box plot, and then excluded from extreme outliers. Then, the descriptive statistics tool of Microsoft Office 2016 Excel was used to analyze the arithmetic mean,$\bar{x}$, and the standard deviation, s, of the sample signal. According to the mean and standard deviation, we obtained data with the confidence interval of 99% ($\bar{x} - 2.58 \times s, \bar{x} + 2.58 \times s$). The signal greater than 99% confidence interval was classified as HPV-positive, and the signal within 99% confidence interval was classified as HPV-negative. In addition, group statistics were performed according to the cancer stage, age, and sex of oral cancer patients and non-oral cancer subjects.

Chapter 3. Results and Discussion

3.1 Synthesis, purification and characterization of HP-3 peptide

The 3D structure of HP-3 peptide was established by utilizing the online Peptide Structure Simulation System PED-FOLD3 to simulate the 3D structure of HP-3 peptide (**Fig. 5**). HP-3 peptide was synthesized on AM resin by using SPPS method. The MALDI-TOF MS was used to characterize the molecular mass of the crude peptide as shown in **Fig. 6**. The molecular mass of HP-3 peptide was detected as 2314.6 m/z which is very close to the theoretical molecular mass of HP-3 peptide (2314.63 Da), demonstrating the success of synthesis of HP-3. The crude mixture of peptides was purified by using RP-HPLC to collect HP-3 peptide, and the chromatograms were shown in **Fig. 7**. The main product HP-3 was eluted at retention time 9.87 min, and the mobile phase solution was approximately 56% DDW (with 0.5% TFA) and 44% ACN (with 0.5% TFA). The collected solution of each peptide was lyophilized using a freeze dryer. After re-dissolving of each dried peptide, the molecular mass and purity were characterized by MALDI-TOF MS. The molecular mass of the purified HP-3 peptide was detected as 2315.8 m/z as shown in **Fig. 8**. The purity of the purified HP-3 peptide was confirmed by using RP-HPLC. As shown in **Fig. 9**, only the signal of our target product HP-3 peptide appeared at the retention time of 9.69 min (the mobile phase solution was approximately 58% DDW with 0.5% TFA and 42% ACN with 0.5% TFA). On the basis of the above results of experiments, we confirm that the designed HP-3 peptide was successfully synthesized.

3.2 Analysis of the specificity of chicken anti-peptide biomarker antibodies

To confirm that the chicken anti-HP-3 (peptide biomarker) antibodies were produced, we performed Western blot to detect the specificity of the self-made antibodies (**Fig. 10**). The uninjected chicken serum was used as the blank control, while the commercial anti-E7 monoclonal antibody was served as the positive control. The molecular mass of the commercial HPV-16 E7 recombinant protein is about 22 kDa, and a specific band was detected at the expected molecular weight as shown in **Fig. 10-B**. Furthermore, to make sure that the band represented the identity of anti-HP-3 antibody, the antibody was premixed with HP-3 peptide for 30 min, followed by the same procedures of Western blot. Since the band for anti-HP-3

antibody disappeared, indicating the specificity of the anti-HP-3 antibody for HP-3 (**Fig. 10-C**). However, we also observed other bands after the membrane reacted with the commercial anti-E7 monoclonal antibody (**Fig. 10-D**). Results showed the multiple molecular mass of the protein that we speculated as the dimer and the trimer of the protein. To prove our speculation, the commercial anti-E7 monoclonal antibody was premixed with E7 protein for 30 min, followed by the same procedures of Western. As shown in **Fig. 10-E**, we successfully removed the dimer and the trimer bands of the protein.

3.3 Biomolecular interaction analysis using the SPR-Biosensor

Using kinetic/affinity analysis to measure the kinetics (association and dissociation rates) and affinity (binding strength) of the ligand in the ligand-analyte interaction. The following experiments study the interactions among anti-E7 monoclonal antibody and E7 protein, anti-HP-3 polyclonal antibody, and HP-3 peptide.

Using Binding Analysis to investigate specific binding of the biomarkers in saliva samples to the E7 or anti-HP-3 antibodies that were immobilized on the CM5 chip surface.

3-3-1 Kinetic and affinity of E7 protein and anti-E7 monoclonal antibody

The result of E7 protein pH scouting shown that pH 4 is the best immobilization pH (**Fig. 11**), and the E7 protein was immobilized onto flow cells 2 successfully (**Fig. 12**). Then used serial dilution of anti-E7 monoclonal antibody as the analyte. Highest concentration is 32 μg/mL, half dilution to 2 μg/mL. It can be observed that the anti-E7 monoclonal antibody caused the ascension in response units (RU), and as the concentration of the anti-E7 monoclonal antibody was injected, the ΔRU also tends to increase (**Fig. 13**). Due to the specificity of the binding between the antibody and the antigen, we chose the "1:1 binding" for kinetic analysis, and calculated the association constant (ka) and the dissociation constant (kd) to be 1.011×10^{5} $M^{-1}s^{-1}$ and 2.314×10^{-3} s^{-1}, respectively. The rest such as equilibrium dissociation constant (KD) are shown in Table 6.Affinity analysis was performed and the signal was increased with dose (**Fig. 14**).

3-3-2 Analysis of E7 and anti-E7 antibody content in saliva samples by binding analysis

In the saliva samples of the following experiments, the "P" represents the patients who have been diagnosed as the third stage of oral cancer, and the "N" represents the non-oral cancer

subject (normal).

The half-dilute saliva samples from 46 oral cancer patients and 34 non-oral cancer subjects were used as analyte to detect anti-E7 antibodies and other substances in saliva, and the sensorgram was detected with the E7 protein immobilized on the chip surface (**Fig. 15** and **Fig. 16**). The results are summarized in Table 7.

Previously, our team have developed the method for detecting osteoporosis-related biomarkers by using surface plasma resonance.[27-28] We also have cooperated with the team of the Oral Surgery Department in Taichung Veterans General Hospital to design a lead peptide as the potential oral cancer biomarkers. Based on these experiences, the SPR based method was used to explore the correlation between biomarkers in saliva and oral cancer and HPV. According to our results, some salivary samples contain substances that binded or interacted to E7 protein (**Fig. 15** and **Fig. 16**). The RUs of E7 protein positive in non-cancer samples (N14, N16, N32, N47 and N71) is less than those of E7 protein positive in patient samples with oral cancer (P31, P37, P49, P55, P79 and P86). Some RUs are also abnormally higher than patient samples with oral cancer. We speculate that the different concentration of saliva samples may cause a false positive reaction. Although the potency of the self-made antibody is not very high, we still can identify the HP-3 positive samples in patient with oral cancer (P27, P29, P31, P33, P35, P37, P43, P59, P63 and P96).

First, exclude extreme values by quartile method (N36 and N51), we can obtain the arithmetic mean is 71.47, the standard deviation is 82.71, and the 99% confidence is 37.66. We conservatively define the signals from saliva samples of oral cancer patients greater than 168.64 is saliva-positive. According the cancer staging (non-cancer, Stage III and Stage IV), age (20-45 years old, 46-60 years old and over 60 years old) and gender (male and female) to do group statistics (**Fig. 17**). The percentage of E7 protein-positive signal from non-cancer saliva sample are 6%, and the percentage of E7 protein-positive signal from oral cancer saliva sample are 8%.

3-3-3 Kinetic and affinity of HP-3 peptide and anti-HP-3 polyclonal antibody

The result of anti-HP-3polyclonal antibody pH scouting shown that pH 4.5 is the best immobilization pH (**Fig. 18**), and the anti-HP-3polyclonal antibody was immobilized onto flow cells 2 successfully (**Fig. 19**). Then used serial dilution of HP-3 peptide as the analyte. Highest concentration is 16 μg/mL, half dilution to 1 μg/mL. It can be observed that the HP-3 peptide caused the ascension in response units (RU), and as the concentration of the HP-3 peptide was

injected, the ΔRU also tends to increase (**Fig. 20**). Due to the specificity of the binding between the antibody and the antigen, we chose the "1:1 binding" for kinetic analysis, and calculated the association constant (ka) and the dissociation constant (kd) to be 6.873×10^{4} $M^{-1}s^{-1}$ and 2.092×10^{-3} s^{-1}, respectively. The rest such as equilibrium dissociation constant (KD) are shown in Table 8. Affinity analysis was performed and the signal was increased with dose (**Fig. 21**).

3-3-4 Analysis of HP-3 and anti-HP-3 antibody content in saliva samples by binding

Analysis

The half-dilute saliva samples from 46 oral cancer patients and 34 non-oral cancer subjects were used as analyte to detect E7 protein and other substances in saliva, and the sensorgram was detected with the anti-HP-3polyclonal antibody immobilized on the chip surface (**Fig. 22** and **Fig. 23**). The results are summarized in Table 9.

First, exclude extreme values by quartile method (N16, N18, N32, and N75), we can obtain the arithmetic mean is -6.01, the standard deviation is 8.31, and the 99% confidence is 3.906. We conservatively define the signals from saliva samples of oral cancer patients greater than 4.067 is saliva-positive. According the cancer staging (non-cancer, Stage III, and Stage IV), age (20-45 years old, 46-60 years old, and over 60 years old) and gender (male and female) to do group statistics (**Fig. 24**). The percentage of HP-3 peptide-positive signal from non-cancer saliva sample are 0%, and the percentage of HP-3 peptide-positive signal from oral cancer saliva sample are 14%.

Chapter 4. Conclusion

In this study, the HP-3 peptide was prepared successfully by the solid phase peptide synthesis, characterized and confirmed by RP-HPLC and MALDI-TOF MS. HP-3 peptide effectively immunized chickens and produced its polyclonal antibodies which are useful for further development of screening products.

Prepared HP-3 peptide and anti-HP-3 polyclonal antibodies have been used for development of biosensor based methods for screening oral cancer patients with HPV infection. As SPR–biosensor has the advantages such as less sample amount, fast detection, real-time analysis, and label-free of any binding partners, our established methods and approaches are promising for further applications in detection of disease biomarkers and tracking the prognosis of patients. In the future, we plan to increase the titer of polyclonal antibodies and to perform PCR analysis to obtain some golden standard data for developing non-invasive immunoassays for oral cancers.

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Appendix

Table 1. The reagents, instruments, and manufacturers for performing SPPS

Reagents and instruments	**Manufacturers**
Column PD-10, Empty	GE Healthcare，USA
Rink Amide AM resin (200-400 mesh)	Merck，Germany
N_{α}-Fmoc-D-Amino Acids	AnaSpec，USA
HBTU, anhydrous	AgeneMax，Taiwan, R.O.C.
HOBt, anhydrous	AgeneMax，Taiwan, R.O.C.
N-Ethyldiisopropylamine	Alfa Aesar，USA
Dichloromethane	Macron Fine Chemicals，USA
N,N-Dimethylformamide	DUKSAN，South Korea
Piperidine	Merck，Germany
Trifluoroacetic acid	Merck，Germany
Ethoxyethane	Sigma，USA
Nitrogen Compressed Gas	Toyo Gas，Taiwan, R.O.C.
Parafilm M	Bemis，USA
4°C Refrigerator : A750R	FuKang，Taiwan, R.O.C.
-20 °C Upright freezer : FFFU21M1QW	FRIGIDAIRE，USA
-80 °C Freezer : VT-208MT	Nihon Freezer，Japan
Freeze Dryer : FD3-12P-80	KingMech Co.,LTD，Taiwan, R.O.C.

Table 2. The reagents, instruments, and manufacturers used by RP-HPLC and MALDI-TOF-MS

Reagents and instruments	**Manufacturers**
Reversed phase high performance liquid chromatography : Pump L-2130	Hitachi, Ltd.，Japan
Reversed phase high performance liquid chromatography : UV Detector L-2130	Hitachi, Ltd.，Japan
Ascentis® C18 HPLC Column (25 cm × 10 mm, 5 μm)	Supelco，USA
Microliter Syringes	Hamilton，USA
Smart Synergy-Type I Ultrapure water system	Merck，Germany
Durapore® Membrane Filter (0.22 μm GV)	Millipore，USA
Acetonitrile	J.T.Baker，USA
Trifluoroacetic acid	Merck，Germany
Ultrasound cleaning machine : FT-GU09	Finetech Research & Innovaion Corp.，Taiwan, R.O.C.
TERUMO® SYRINGE with NEEDLE (1 cc)	Terumo，Japan
PureTech® Syringe Filter (0.22 μm)	Widetron Technologies Corp.，Taiwan, R.O.C.
Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry : microflex	Bruker，Germany
α-Cyano-4-hydroxycinnamic acid	Aldrich，USA
Acetonitrile	J.T.Baker，USA
Trifluoroacetic acid	Merck，Germany

Table 3. The reagents, instruments, and manufacturers used by preparation of chicken anti-peptide biomarker antibodies

Reagents and instruments	**Manufacturers**
Hens : Arbor Acres Plus	Professor Borrung Ou from the Department of Animal Science and Biotechnology of Tunghai University
Needle	TOP SURGICAL Taiwan Co., Ltd
Freund's Adjuvant, Complete	Sigma-Aldrich，USA
Freund's Adjuvant, Incomplete	Sigma-Aldrich，USA
HP-3 peptide	Kelowna International Scientific Inc.，Taiwan, R.O.C.
HP-3 peptide conjugated KLH	Kelowna International Scientific Inc.，Taiwan, R.O.C.
Chloroform	Mallinckrodt，USA
Centrifuge : 5415 D	Eppendorf，Germany
Centrifuge: himac CR5B	Hitachi, Ltd.，Japan

Table 4. The reagents, instruments, and manufacturers used by Western blot

Reagents and instruments	**Manufacturers**
Rotary oscillating constant temperature incubator: S300	Firstek，Taiwan, R.O.C.
Rotary oscillating constant temperature incubator: OS-701	TKS Orbital Shaker，Taiwan, R.O.C.
iBlot™ Transfer Stack, PVDF, regular size : IB401001	Invitrogen，USA
iBlot® Dry Blotting System : IB1001	Invitrogen，USA
PageRuler™ Prestained Protein Ladder, 10 to 180 kDa : 26616	Thermo Fisher Scientific，USA
20X Bolt™ MES SDS Running Buffer : B0002	Invitrogen，USA
Bolt™ 4-12% Bis-Tris Plus Gels, 15-well : NW04125BOX	Invitrogen，USA
Bolt™ 4-12% Bis-Tris Plus Gels, 10-well : NW04120BOX	Invitrogen，USA
Anchor nonfat dry milk	Fonterra Brands (Far East) Limited，Taiwan, R.O.C.
TWEEN® 20 : P9416	SIGMA，USA
5X SDS-PAGE Protein Sample Buffer : W-5XPD	GoalBio Hycell international Co. Ltd.，

TMB Chromogen Solution (for Western Blot)	Thermo Fisher Scientific，USA
Recombinant HPV16 E7 Protein : CSB-EP365855HML	Cusabio，China
HP-3 peptide	Kelowna International Scientific Inc.，Taiwan, R.O.C.
HPV16 (E7) antibody : orb24091	Biorbyt，UK
Anti-HPV 16 (E7) antibody : [TVG 701Y] ab20191	Abcam，UK
Anti-6X His Tag Mouse Monoclonal Antibody : PT-6XHis-100ug	Protech Technology Enterprise Co., Ltd.，Taiwan, R.O.C.
Goat anti-Mouse IgG (H+L) Secondary Antibody, HRP Conjugate (Catalog# 31430)	Thermo Fisher Scientific，USA
Goat anti-Chicken IgY (H+L) Secondary Antibody, HRP Conjugate (Catalog# A16054)	Thermo Fisher Scientific，USA

Table 5. The reagents, instruments, and manufacturers used by SPR

Reagents and instruments	Manufacturers
Biacore X100	GE Healthcare，USA
Aquaplore 3S - AIC Water	Aquapro International Company，Taiwan, R.O.C.
Sensor Chip CM5	GE Healthcare，USA
Amine Coupling Kit (BR-1000-50)	GE Healthcare，USA
1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride	Sigma-Aldrich，USA
N-Hydroxysuccinimide	Alfa Aesar，USA
Ethanolamine hydrochloride	ACROS，Belgium
BIA maintenance Kit (BR-1006-66)	GE Healthcare，USA
HBS-EP+ buffer, 10X (BR-1008-26)	GE Healthcare，USA
Recombinant HPV16 E7 Protein : CSB-EP365855HML	Cusabio，China
HP-3 peptide	Kelowna International Scientific Inc.，Taiwan, R.O.C.

Table 6. Kinetic analysis of E7 protein and anti-E7 monoclonal antibodies at different concentrations

k_a (1/Ms)	k_d (1/s)	K_D (M)	R_{max} (RU)	tc	Chi^2 (RU^2)
1.011×10^5	0.002314	2.289×10^{-8}	5333	1.030×10^7	6.94×10^4

Table 7. Statistical analysis of negative and positive grouping of saliva samples against E7 protein

Characteristics	E7 protein-positive (N=11)	E7 protein-negative (N=67)
Stage		
Normal	5 (6%)	27 (35%)
stage III	0 (0%)	17 (22%)
stage IVA	6 (8%)	23 (29%)
Age		
Adults (20-45)	5 (6%)	29 (37%)
Middle age (46-60)	4 (5%)	24 (31%)
Old (>60)	2 (3%)	14 (18%)
Sex		
Male	7 (9%)	52 (67%)
Female	4 (5%)	15 (19%)

Table 8. Kinetic analysis of Anti-HP-3 polyclonal antibody and HP-3 peptide at different concentrations

k_a (1/Ms)	k_d (1/s)	K_D (M)	R_{max} (RU)	tc	Chi^2 (RU^2)
6.873×10^4	0.002092	3.043×10^{-8}	0.007342	1.000×10^{-8}	10.5

Table 9. Statistical analysis of negative and positive grouping of saliva samples against HP-3 peptide

Characteristics	HP-3 peptide-positive (N=10)	HP-3 peptide-negative (N=66)
Stage		
Normal	0 (0%)	29 (38%)
stage III	2 (3%)	16 (21%)
stage IVA	8 (11%)	21 (28%)
Age		
Adults (20-45)	2 (3%)	31 (41%)
Middle age (46-60)	3 (4%)	25 (33%)
Old (>60)	5 (7%)	10 (13%)
Sex		
Male	9 (12%)	48 (63%)
Female	1 (1%)	18 (24%)

Table 10. Binding analysis of 1/2 diluted saliva samples with CM5 sensor chip immobilized E7 protein.

Stage	NO.	Sex	Age	$\Delta RU_{fin.}$	Stage	NO.	Sex	Age	$\Delta RU_{fin.}$
Normal	N12	F	26	49.471	Normal	N71	F	23	262.198
Normal	N14	M	41	183.153	Normal	N73	F	41	31.820
Normal	N16	M	62	190.345	Normal	N75	M	42	15.031
Normal	N18	F	24	7.884	Normal	N77	M	28	140.064
Normal	N20	M	43	54.050	Normal	N79	F	47	30.145
Normal	N22	M	52	35.137	Normal	N81	M	46	104.955
Normal	N24	M	50	27.095	stage IVA	P21	M	52	7.616
Normal	N28	F	31	44.558	stage IVA	P23	M	59	1.309
Normal	N30	M	57	161.068	stage IVA	P25	M	53	3.881
Normal	N32	F	56	283.187	stage IVA	P27	M	43	0.896
Normal	N34	F	27	152.572	stage IVA	P29	M	70	30.187
Normal	N36	M	37	67.132	stage IVA	P31	F	48	263.793
Normal	N39	F	50	400.746	stage IVA	P33	M	66	13.205
Normal	N41	F	37	19.553	stage III	P35	M	50	69.852
Normal	N43	F	30	27.170	stage IVA	P37	M	66	199.696
Normal	N45	F	37	28.087	stage IVA	P39	M	64	13.277
Normal	N47	F	26	204.479	stage IVA	P41	M	52	-2.899
Normal	N49	F	23	4.639	stage IVA	P43	M	66	10.250
Normal	N51	M	24	28.199	stage III	P45	M	68	2.669
Normal	N53	F	22	652.853	stage III	P47	M	42	3.936
Normal	N55	M	27	126.842	stage IVA	P49	M	36	173.695
Normal	N57	M	26	-8.133	stage III	P51	M	39	-3.844
Normal	N59	M	20	-0.433	stage III	P53	F	66	13.310
Normal	N61	M	20	-0.448	stage IVA	P55	M	40	327.256
Normal	N63	F	37	0.628	stage IVA	P57	M	59	2.697
Normal	N65	M	21	13.327	stage IVA	P59	M	60	50.753
Normal	N67	M	38	-2.980	stage IVA	P61	M	60	-1.368
Normal	N69	F	32	6.264	stage III	P63	M	68	16.705

Stage	NO.	Sex	Age	$\Delta RU_{fin.}$	Stage	NO.	Sex	Age	$\Delta RU_{fin.}$
stage IVA	P65	M	46	33.603	stage IVA	P94	F	71	135.571
stage III	P67	M	54	1.937	stage IVA	P96	M	45	-0.186
stage III	P69	M	51	11.146	stage IVC	P98	M	48	5.334
stage III	P71	M	79	37.824	stage III	P100	M	52	6.382
stage IVA	P73	M	38	66.393	stage III	P102	M	62	20.911
stage IVA	P75	M	61	29.701	stage IVA	P104	M	36	6.183
stage IVA	P77	M	63	87.312	stage IVA	P106	F	51	131.536
stage IVA	P79	M	60	359.672	stage IVB	P108	M	51	3.088
stage III	P81	M	40	128.585	stage III	P110	M	52	18.334
stage III	P82	M	66	51.349	stage III	P100	M	52	6.382
stage III	P84	M	53	2.124	stage III	P102	M	62	20.911
stage IVA	P86	M	51	251.070	stage IVA	P104	M	36	6.183
stage III	P88	M	60	44.634	stage IVA	P106	F	51	131.536
stage III	P90	M	61	3.389	stage IVB	P108	M	51	3.088
stage IVB	P92	M	60	31.596	stage III	P110	M	52	18.334

Table 11. Binding analysis of 1/2 diluted saliva samples with CM5 sensor chip immobilized anti-HP-3 polyclonal antibody.

Stage	NO.	Sex	Age	$\Delta RU_{fin.}$	Stage	NO.	Sex	Age	$\Delta RU_{fin.}$
Normal	N12	F	26	-3.052	Normal	N71	F	23	-23.984
Normal	N14	M	41	-1.557	Normal	N73	F	41	-0.517
Normal	N16	M	62	36.558	Normal	N75	M	42	-66.672
Normal	N18	F	24	-73.892	Normal	N77	M	28	-1.828
Normal	N20	M	43	-19.444	Normal	N79	F	47	-7.179
Normal	N22	M	52	-3.560	Normal	N81	M	46	-0.685
Normal	N24	M	50	-2.954	stage IVA	P21	M	52	0.541
Normal	N28	F	31	-19.678	stage IVA	P23	M	59	-3.364
Normal	N30	M	57	0.311	stage IVA	P25	M	53	-5.285
Normal	N32	F	56	-45.615	stage IVA	P27	M	43	4.662
Normal	N34	F	27	-1.591	stage IVA	P29	M	70	7.981
Normal	N36	M	37	-19.258	stage IVA	P31	F	48	44.940
Normal	N39	F	50	-16.978	stage IVA	P33	M	66	15.461
Normal	N41	F	37	-4.333	stage III	P35	M	50	17.718
Normal	N43	F	30	-3.554	stage IVA	P37	M	66	162.994
Normal	N45	F	37	0.938	stage IVA	P39	M	64	-8.342
Normal	N47	F	26	-1.813	stage IVA	P41	M	52	-3.282
Normal	N49	F	23	-6.078	stage IVA	P43	M	66	7.755
Normal	N51	M	24	0.834	stage III	P45	M	68	-0.619
Normal	N53	F	22	0.897	stage III	P47	M	42	-0.078
Normal	N55	M	27	0.083	stage IVA	P49	M	36	-0.820
Normal	N57	M	26	-2.985	stage III	P51	M	39	-0.741
Normal	N59	M	20	-1.396	stage III	P53	F	66	0.148
Normal	N61	M	20	-4.377	stage IVA	P55	M	40	-0.025
Normal	N63	F	37	-0.496	stage IVA	P57	M	59	0.228
Normal	N65	M	21	-1.523	stage IVA	P59	M	60	23.224
Normal	N67	M	38	-5.269	stage IVA	P61	M	60	-0.764
Normal	N69	F	32	-29.316	stage III	P63	M	68	9.465

Stage	NO.	Sex	Age	$\Delta RU_{fin.}$	Stage	NO.	Sex	Age	$\Delta RU_{fin.}$
stage IVA	P65	M	46	-0.649	stage III	P88	M	60	-14.418
stage III	P67	M	54	-17.441	stage III	P90	M	61	-5.431
stage III	P69	M	51	0.675	stage IVB	P92	M	60	-0.020
stage III	P71	M	79	-3.798	stage IVA	P94	F	71	-0.796
stage IVA	P73	M	38	-0.144	stage IVA	P96	M	45	10.132
stage IVA	P75	M	61	0.403	stage IVC	P98	M	48	-10.534
stage IVA	P77	M	63	-1.017	stage III	P100	M	52	-1.339
stage IVA	P79	M	60	-11.678	stage III	P102	M	62	-17.688
stage III	P81	M	40	3.334	stage IVA	P104	M	36	-2.407
stage III	P82	M	66	-3.280	stage IVA	P106	F	51	-15.823
stage III	P84	M	53	-29.687	stage IVB	P108	M	51	-3.758
stage IVA	P86	M	51	-5.445	stage III	P110	M	52	-0.695

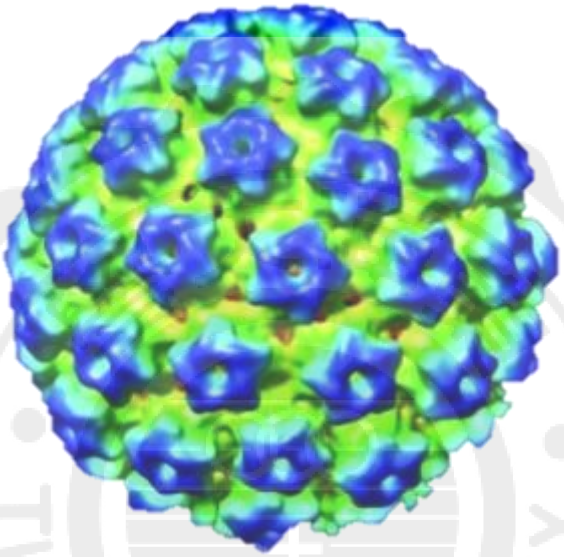

Figure 1. 3D model of HPV[29]

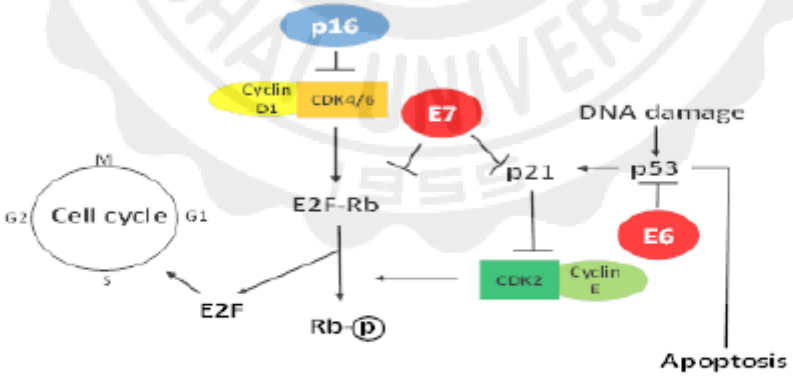

Figure 2. Signaling pathways of high risk HPV oncogenes[6]

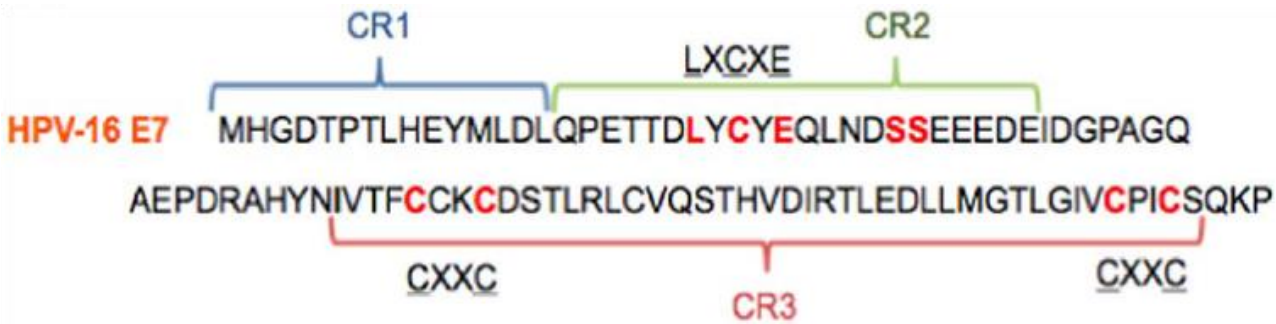

Figure 3. HPV-16 E7 protein conserved region[14]

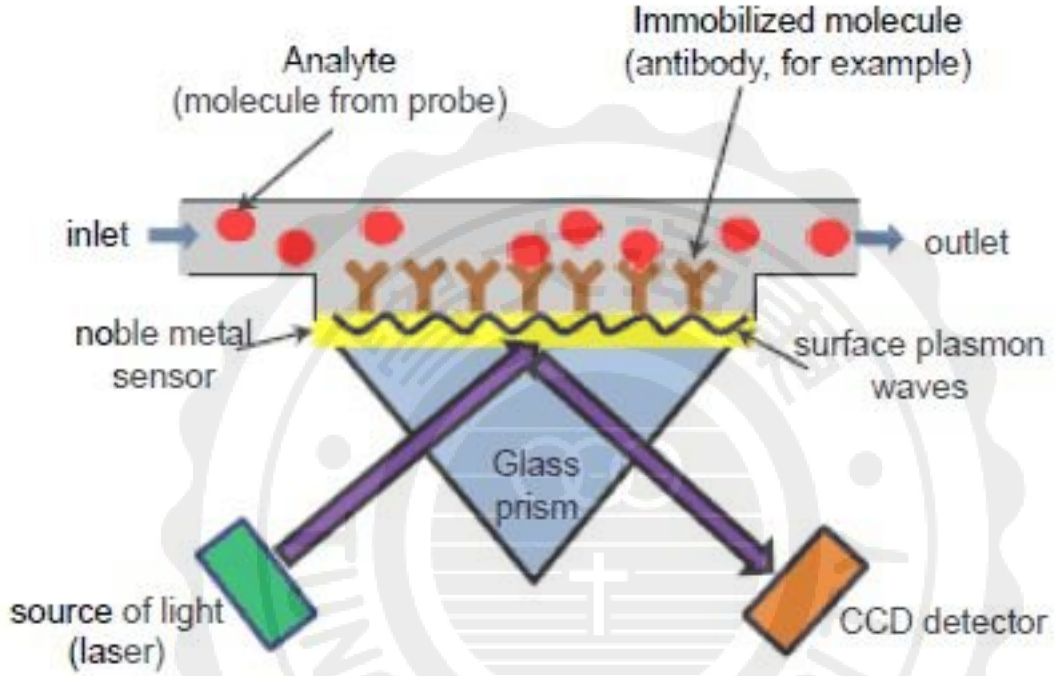

Figure 4. SPR Detection principle[17]

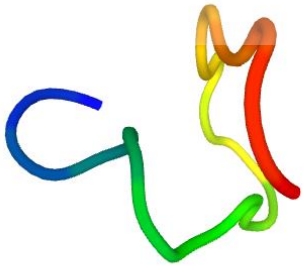

Figure 5. 3D structure of the HP-3 peptide

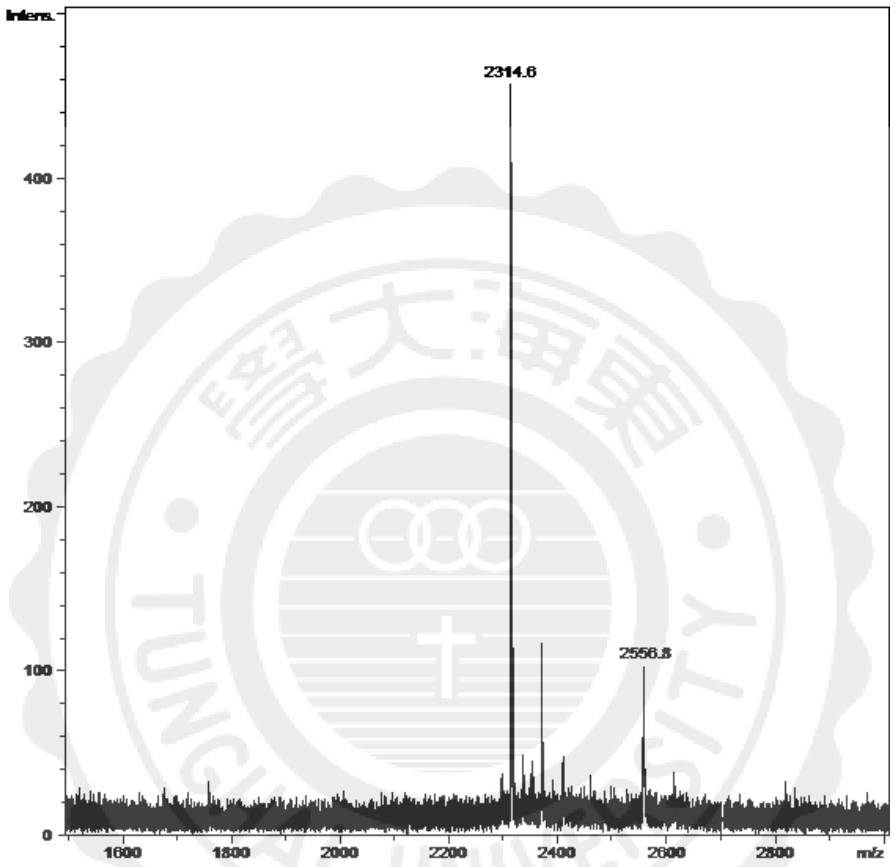

Figure 6. Mass spectra of the HP-3 crude peptide.

Molecular mass detected for the crude mixture of peptides, containing the HP-3 peptide with the detected mass of 2314.6 Da.

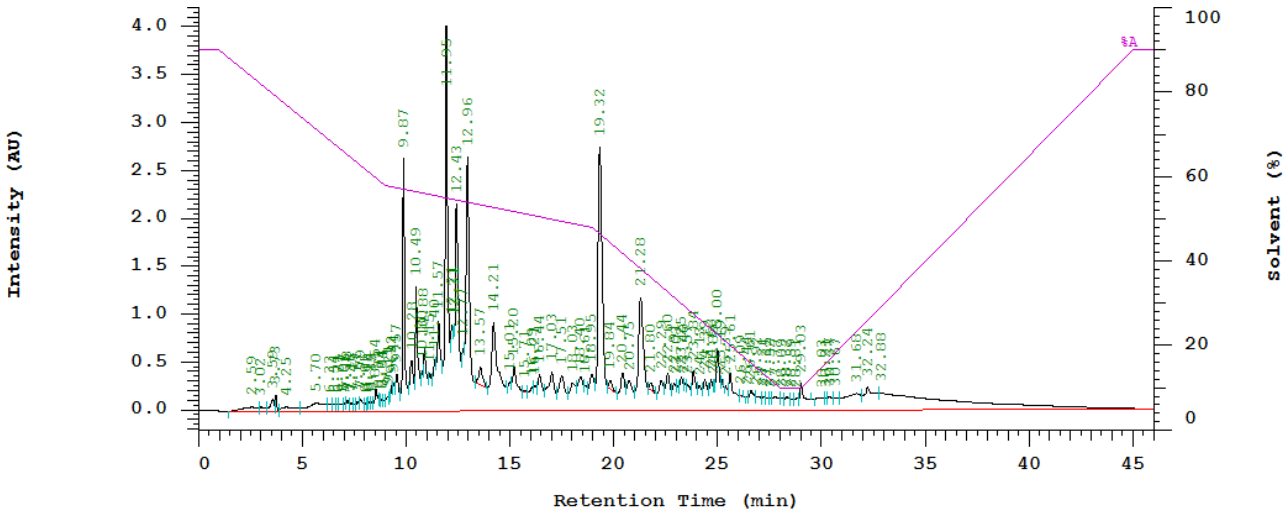

Figure 7. RP-HPLC chromatogram of the HP-3 crude peptide

Retention time of the HP-3 peptide in the crude mixture of peptides was determined as 9.87 minutes.

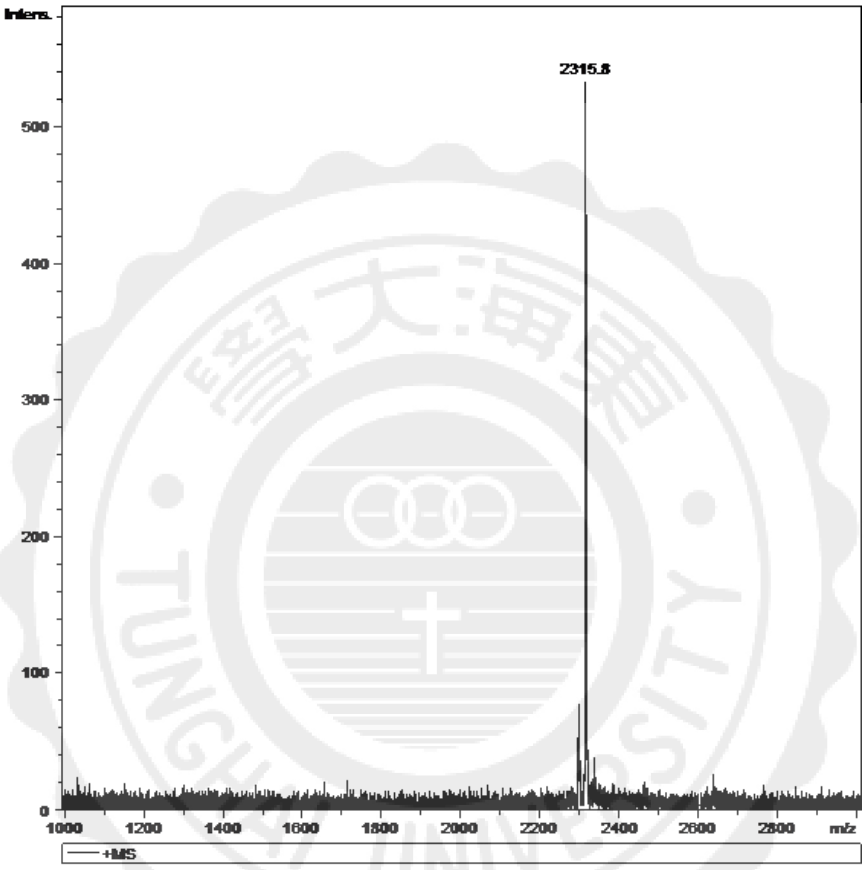

Figure 8. Mass spectra of the HP-3 pure peptide

Molecular mass of the purified HP-3 peptide was determined as 2315.8 Da.

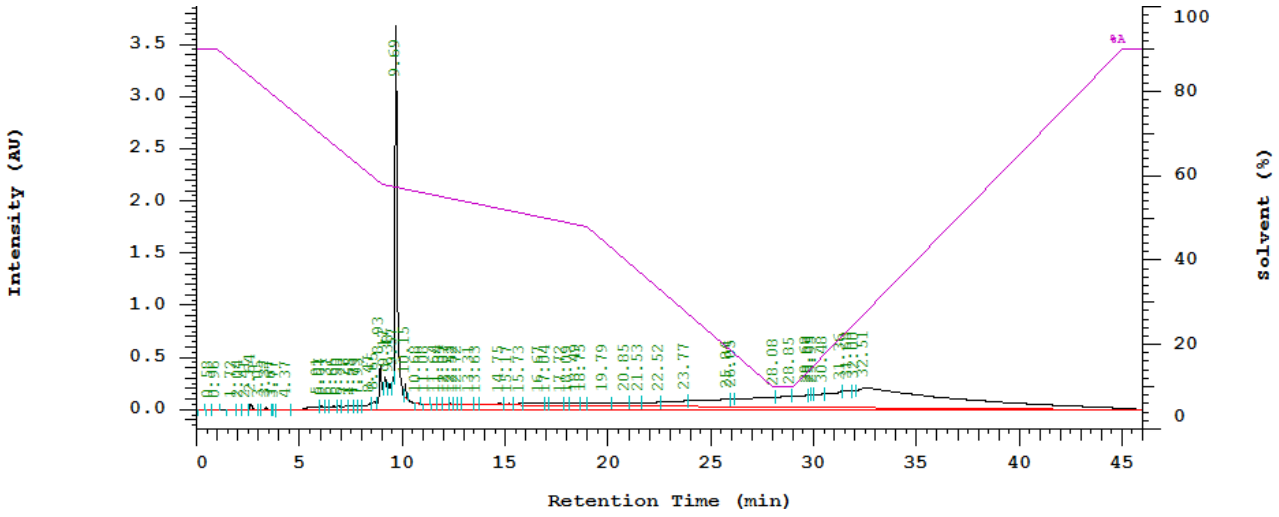

Figure 9. RP-HPLC chromatogram of the purified HP-3 peptide.

Retention time of the purified HP-3 peptide was determined as 9.69 minutes.

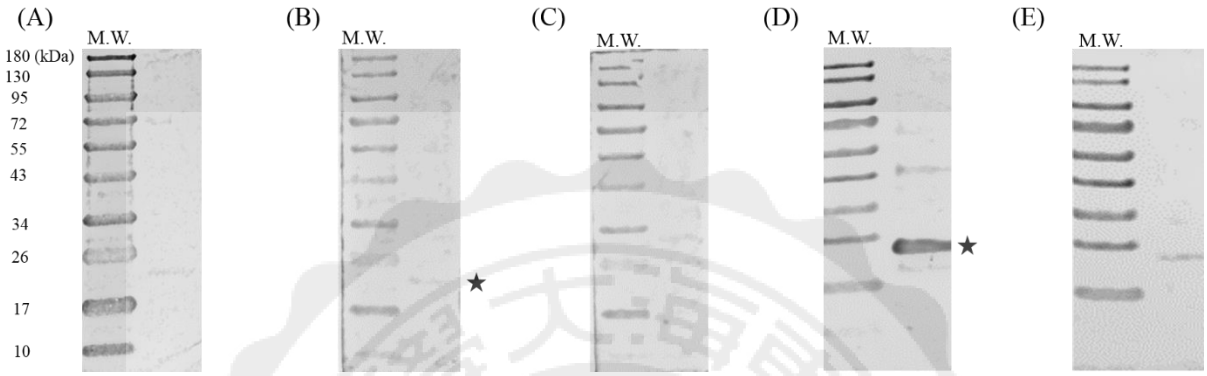

Figure 10. Western blot analysis. Western blot analysis of the specificity of different antibodies to HPV-16 E7 protein. The primary antibody is (A) control chicken serum with 250X dilution, (B) Anti-HP-3 chicken serum with 50X dilution, (C) Anti-HP-3 chicken serum with 50X dilution premixed with HP-3 peptide for 30 minutes, (D) Anti-E7 monoclonal antibody with 1000X dilution, or (E) Anti-E7 monoclonal antibody with 1000X dilution premixed with E7 protein for 30 minutes.

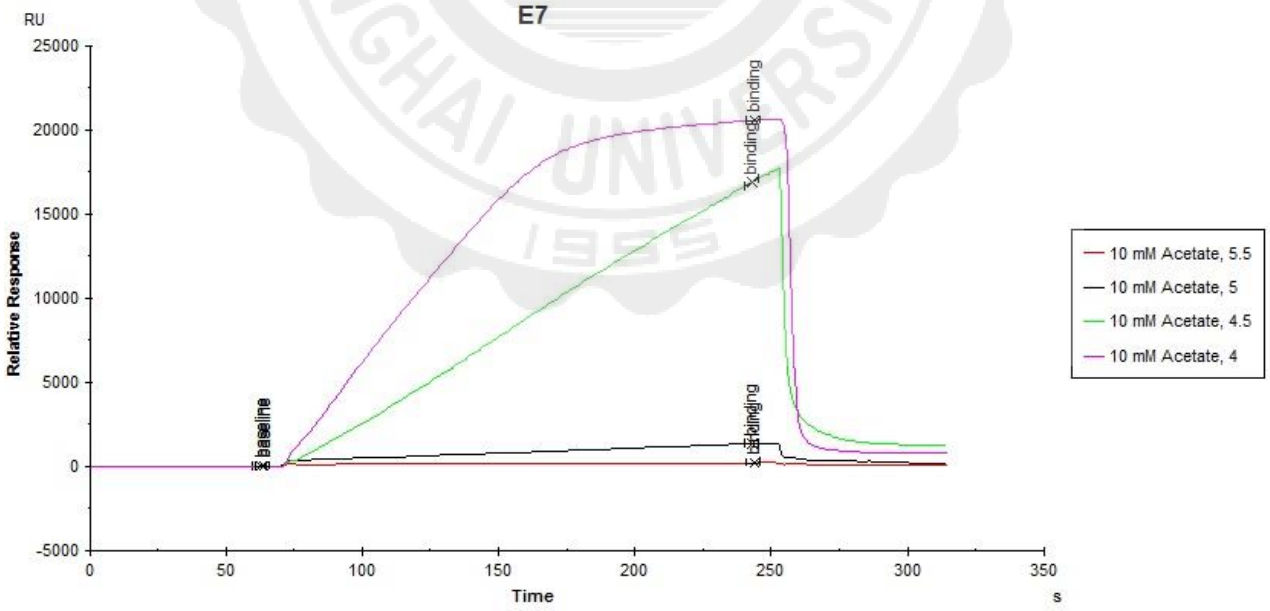

Figure 11. Sensorgrams for optimizing the condition of immobilization of E7 protein.
Using different pH (pH=4、pH=4.5、pH=5、pH=5.5) of 10 mM sodium acetate as dilution buffer, pH 4 is the best immobilization pH.

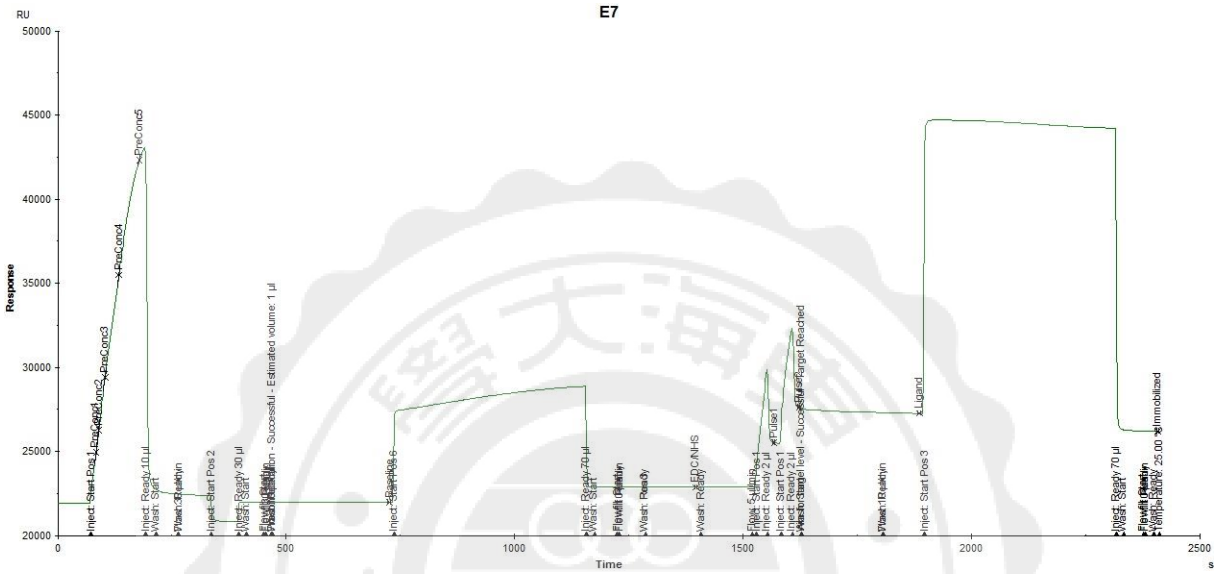

Figure 12. Sensorgrams of immobilization of E7 protein on the sensor chip.

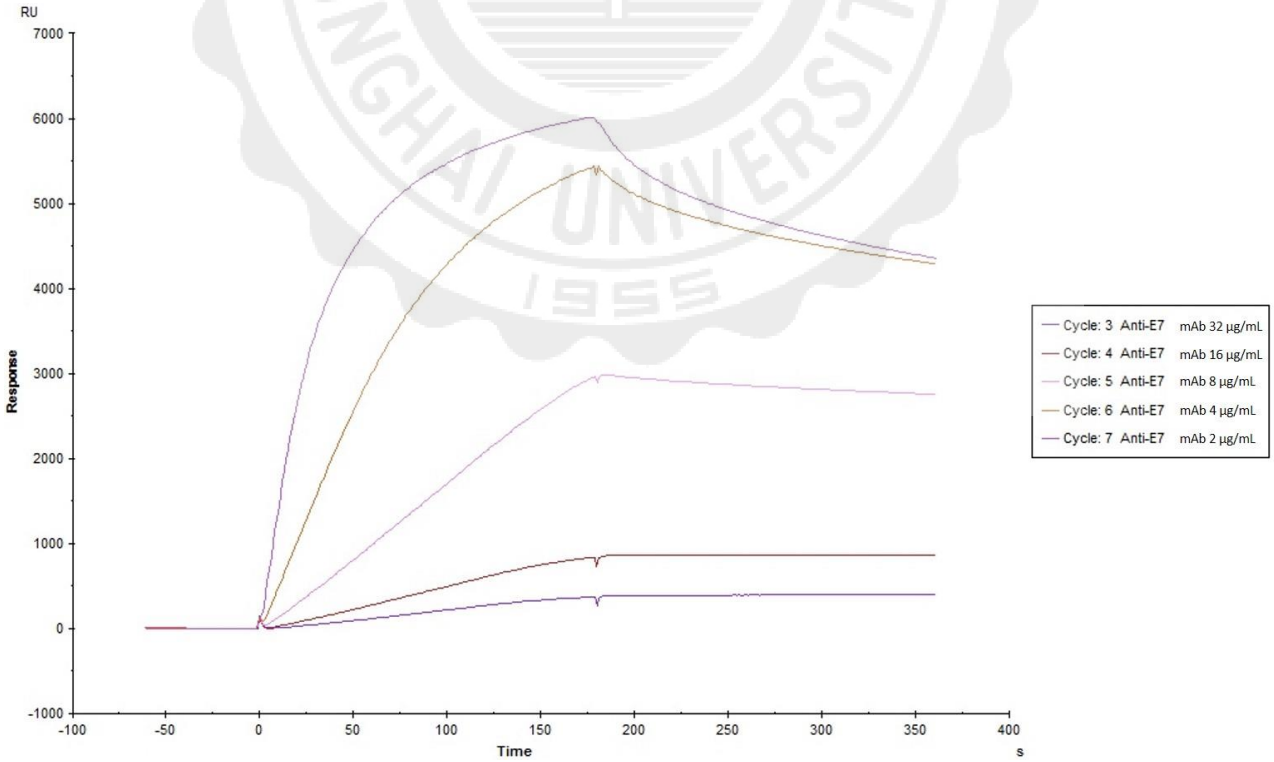

Figure 13. Kinetic analysis of E7 protein and anti-E7 monoclonal antibody.

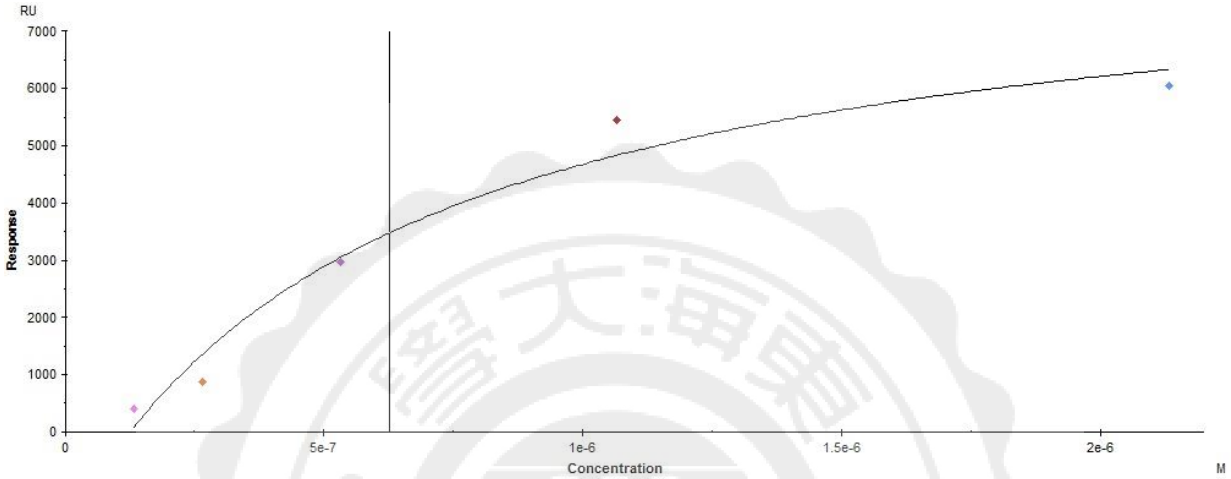

Figure 14. Affinity analysis of E7 protein and different concentration of anti-E7 monoclonal antibody (2、4、8 、16 、32 μg/mL).

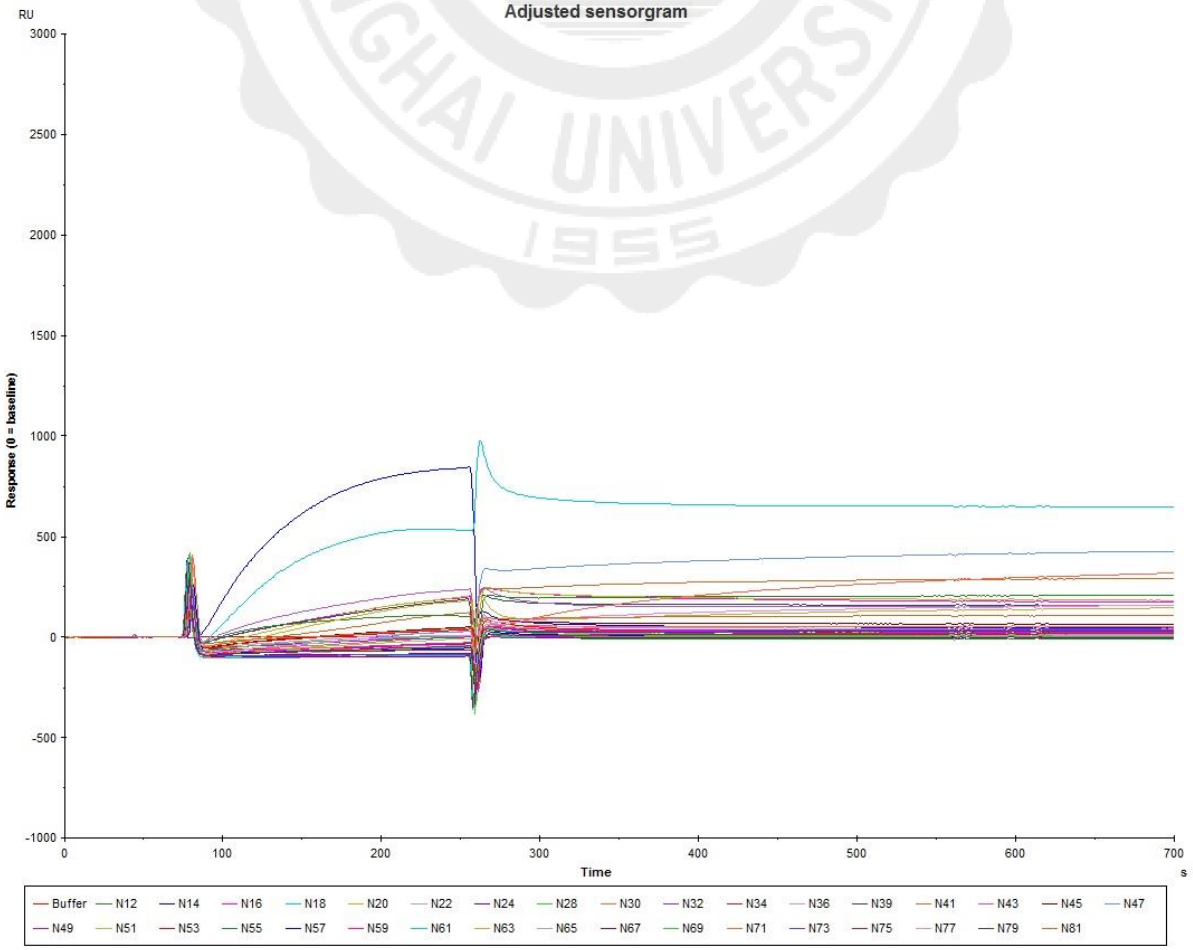

Figure 15. Sensorgrams of the interaction of E7 protein with non-cancer saliva samples.

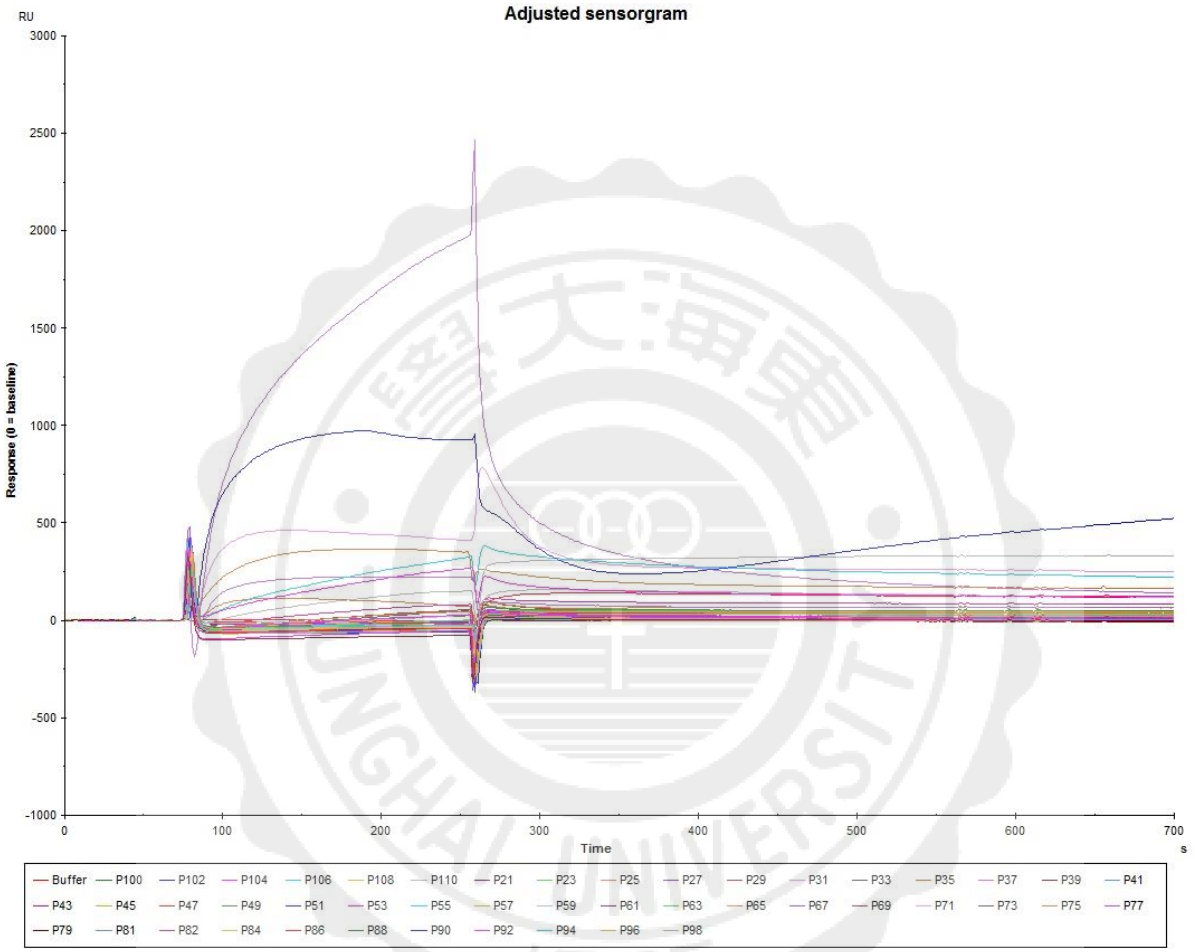

Figure 16. Sensorgrams of the interaction of E7 protein with oral cancer saliva samples.

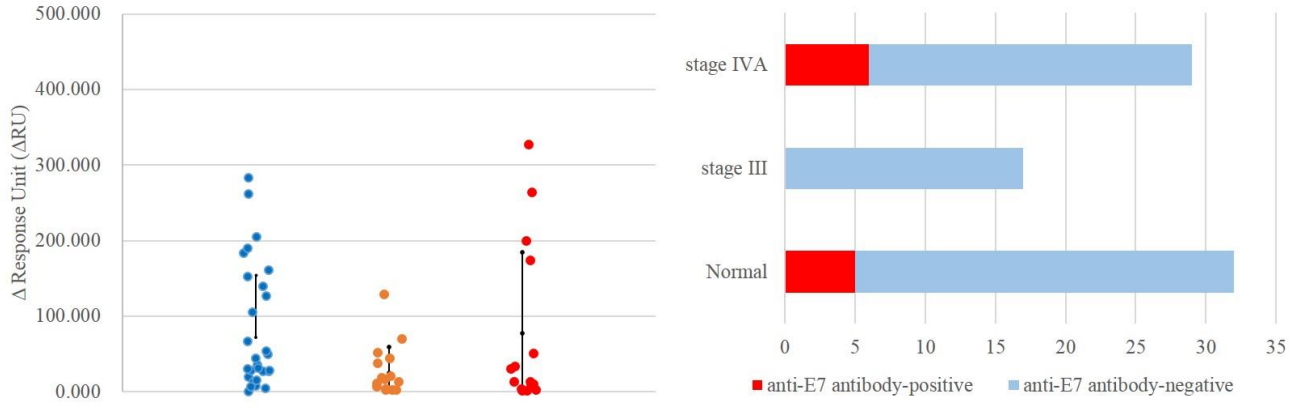

Figure 17. Statistical analysis of the affinity of E7 protein and saliva samples.

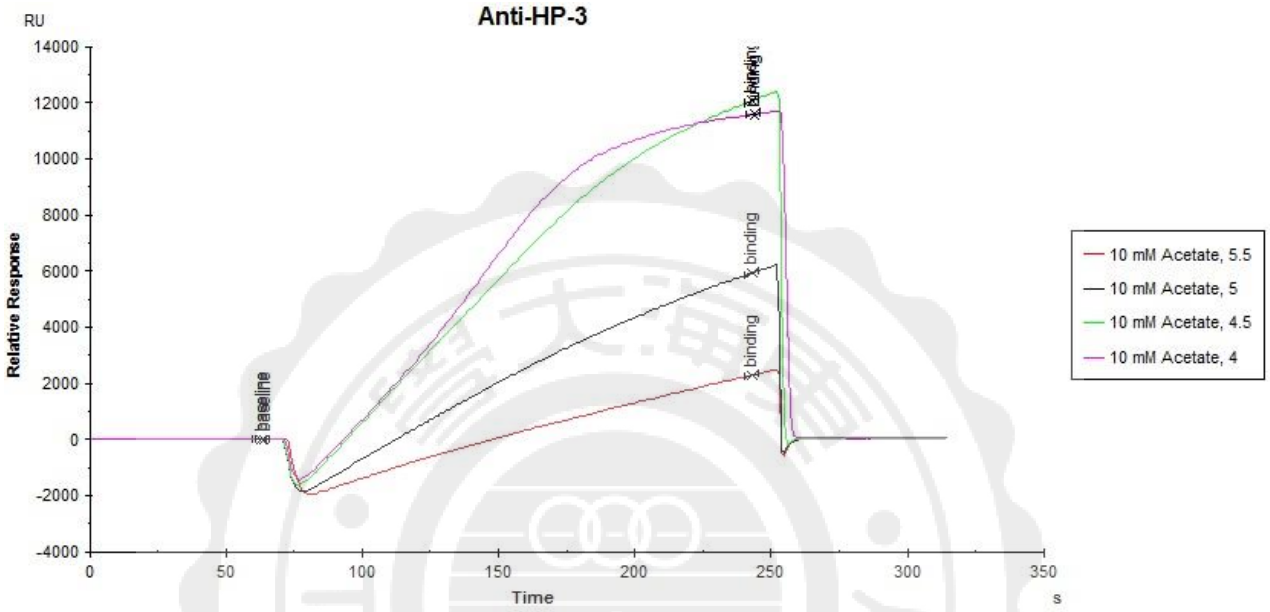

Figure 18. Sensorgrams for optimizing the condition of immobilization of anti-HP-3 pAb. Using different pH (pH=4、pH=4.5、pH=5、pH=5.5) of 10 mM sodium acetate as dilution buffer, pH 4.5 is the best immobilization pH.

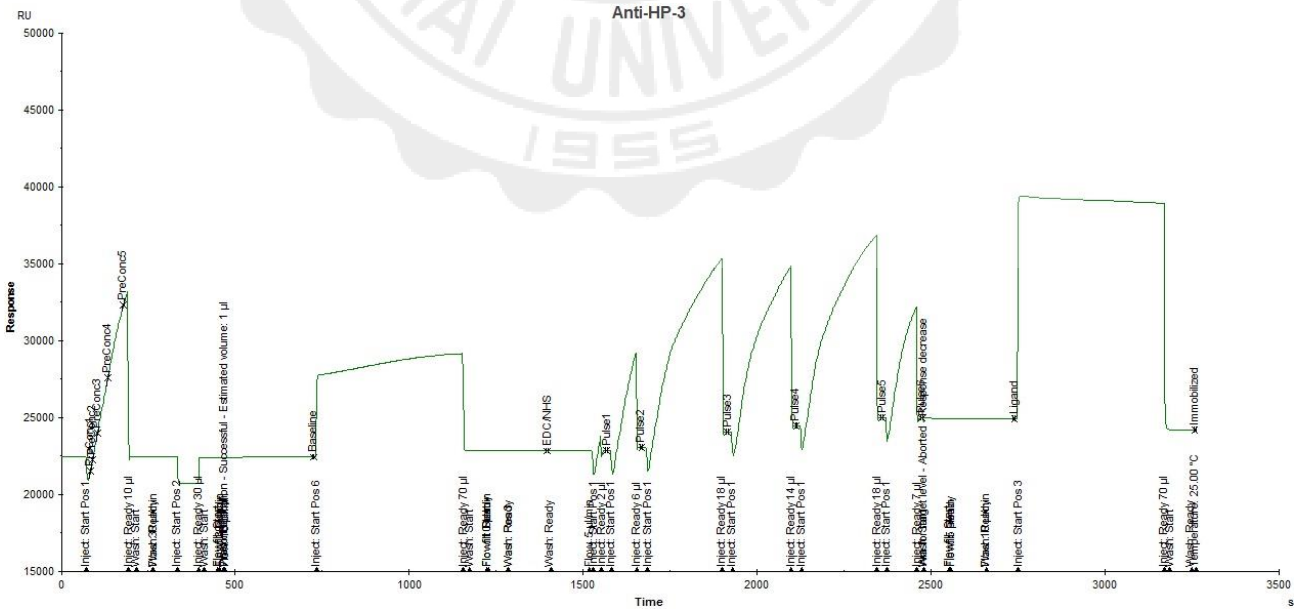

Figure 19. Sensorgrams of immobilization of anti-HP-3 pAb on the sensor chip.

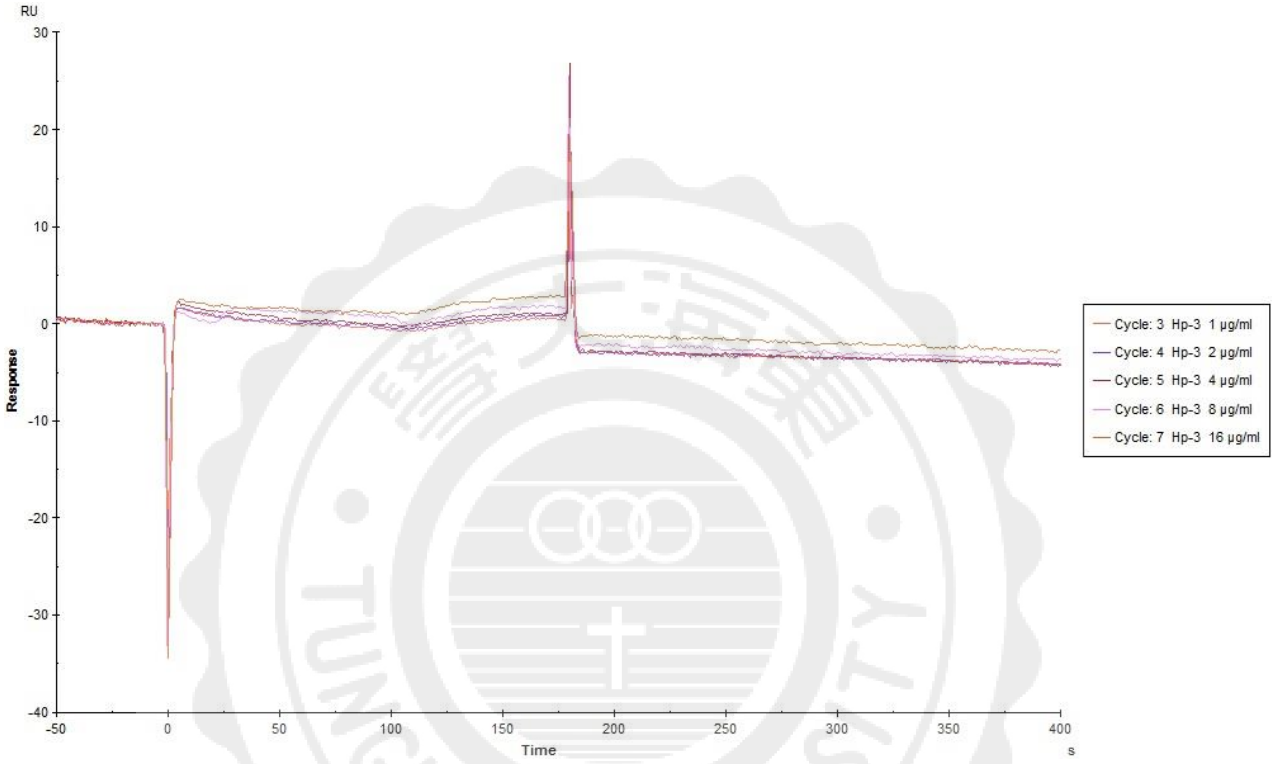

Figure 20. Kinetic analysis of anti-HP-3 polyclonal antibody and HP-3 peptide.

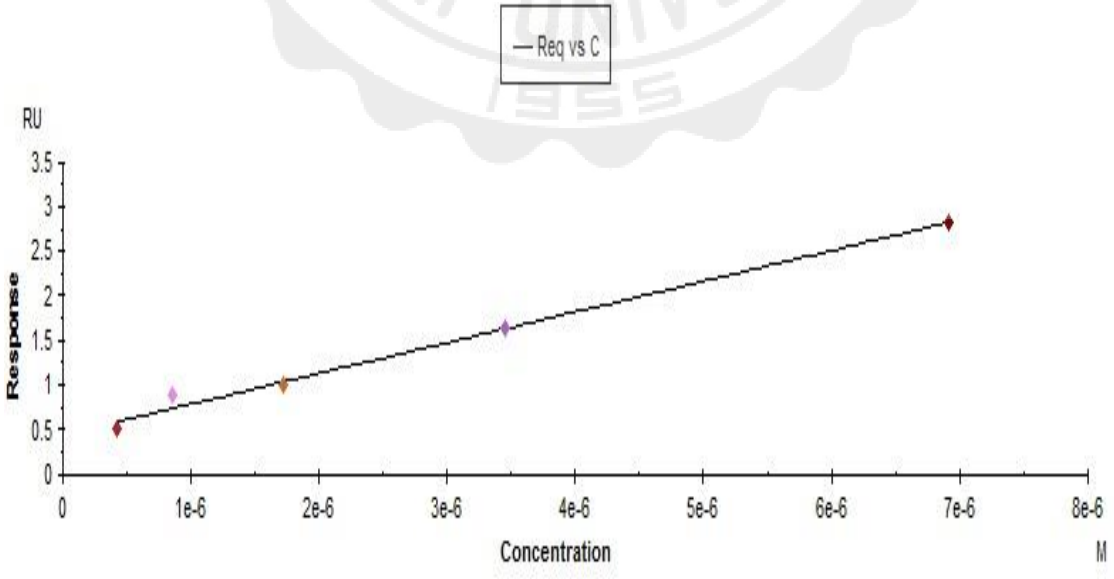

Figure 21. Affinity analysis of anti-HP-3 polyclonal antibody and different concentration of HP-3 peptide (1、2、4、8、16 µg/mL).

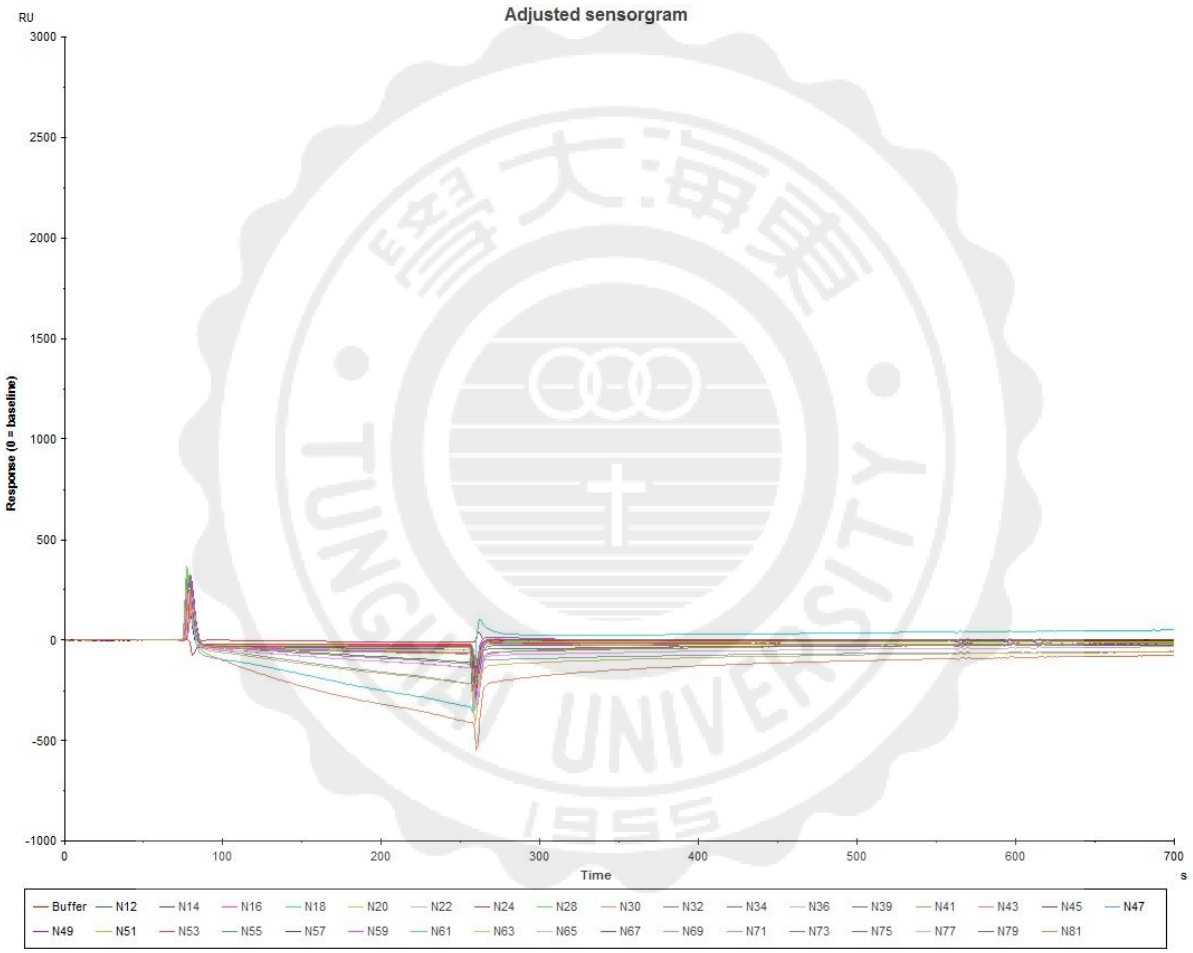

Figure 22. Sensorgrams of the interaction of anti-HP-3 pAb with non-cancer saliva samples.

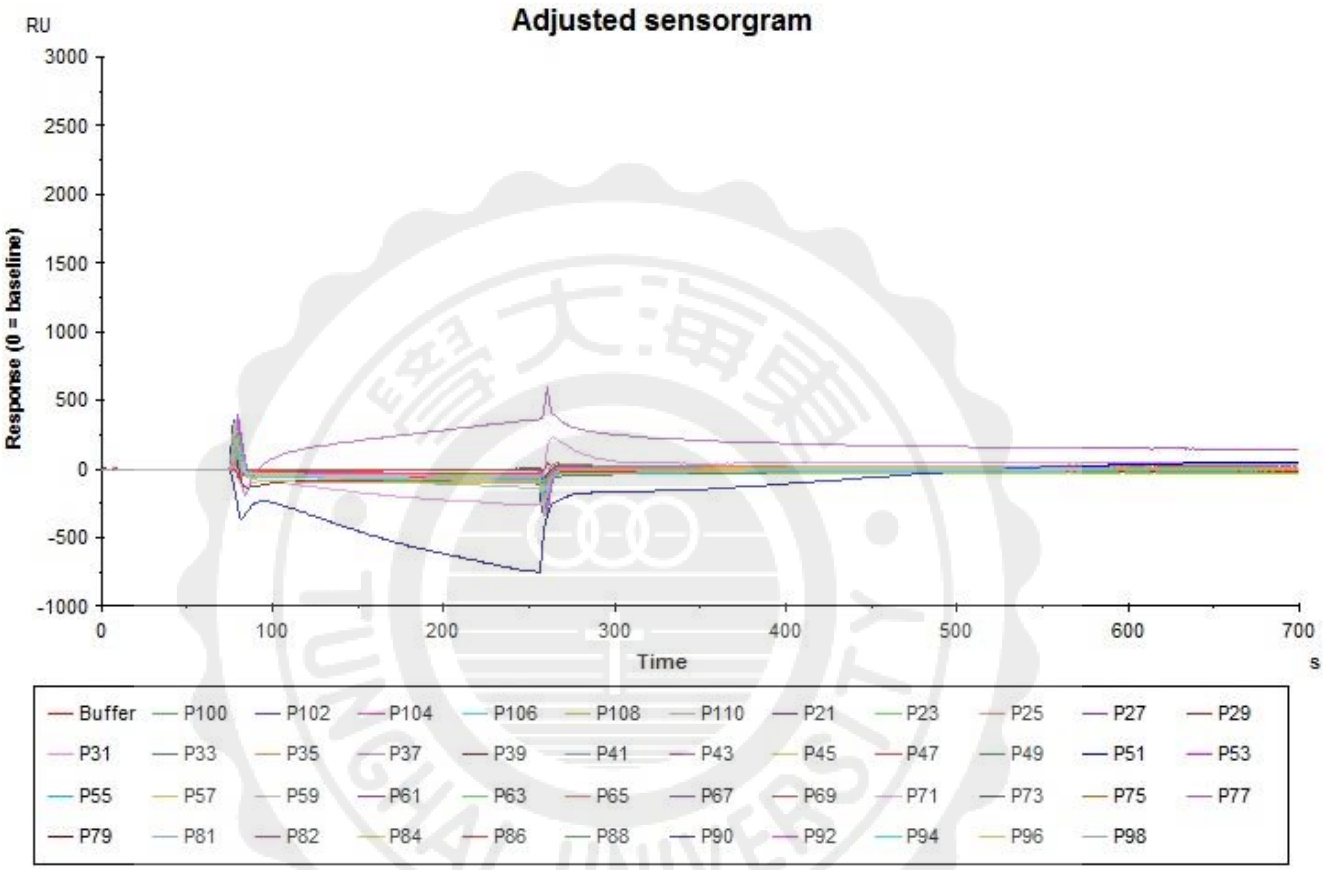

Figure 23. Sensorgrams of the interaction of anti-HP-3 pAb with oral cancer saliva samples.

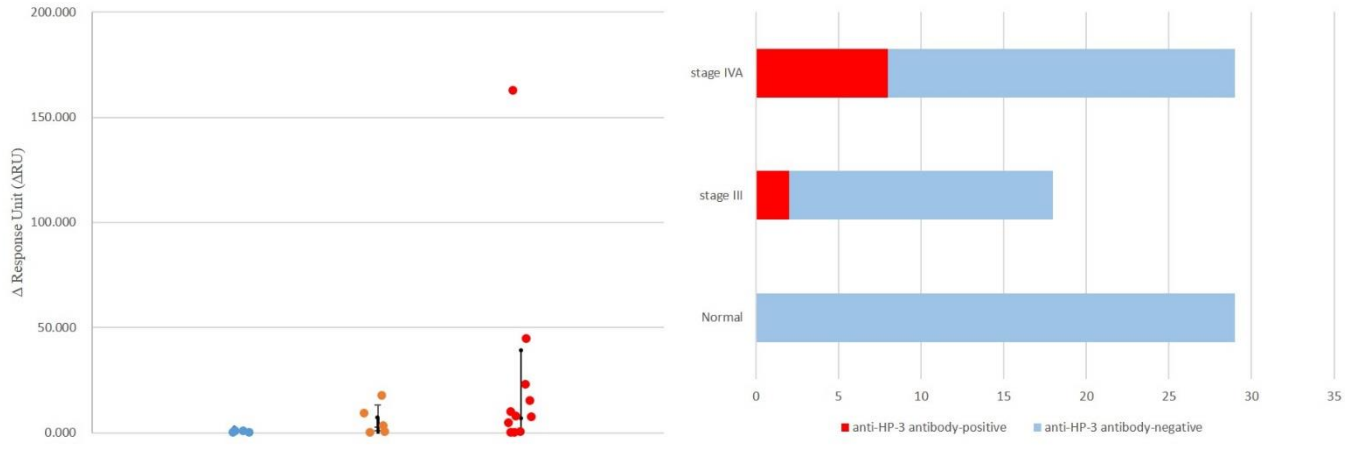

Figure 24. Statistical analysis of the affinity of Anti-HP-3 pAb and saliva samples.