Optical Detection of Human Papillomavirus Type 16 and Type 18 by Sequence Sandwich Hybridization With Oligonucleotide-Functionalized Au Nanoparticles

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***Abstract*—The importance of detecting and subtyping human papillomaviruses (HPVs) in clinical and epidemiological studies has been well addressed. In detecting the most common types of HPV, type 16 (HPV-16) and type 18 (HPV-18), in the cervical mucous of patients in a simple and rapid manner, the assay of a label-free colorimetric DNA sensing method based on sequence sandwich hybridization with oligonucleotide-functionalized Au nanoparticles (AuNPs) was fabricated in this study. Specific oligonucleotide probes were designed for the sequence detection within the L1 gene of HPV-16 and HPV-18, and the probes were capped onto AuNPs, as AuNP probes. The target HPV sequences in clinical specimens were obtained by an asymmetric polymerase chain reaction (PCR) with universal primers, which can amplify the target sequences from several HPV serotypes, including HPV-16 and HPV-18. The DNA sandwich hybridization between the target sequences and the specific AuNP probes was performed at a temperature closer to the theoretical melting temperature of the DNA hybridization. Next, the procedure of increasing salt concentration and cooling the hybridizing solution was immediately utilized to discriminate the target sequences of HPV-16 or HPV-18. If the target sequences were not complementary to sequences of AuNP probes, the AuNPs would aggregate because no duplex DNA formation occurred such that the color of the reaction solution changed from red to purple. If the AuNP probes were a perfect match to the target sequences and a full DNA sandwich hybridization occurred, the reaction solution maintained its red color. A total of 70 mucous specimens from patients with cervical intraepithelial neoplasia were tested by the AuNP probes sandwich hybridization. The results show that there were 33, 16, 5, and 16 cases detected with HPV-16, HPV-18, both HPV-16 and HPV-18 (HPV-16/HPV-18), and neither HPV-16 nor HPV-18, respectively. In comparison with the specific detection by TaqMan real-time PCR assays for HPV-16, the detection sensitivity and specificity of the AuNP probes sandwich hybridization reached 95% and 90%, respectively, for HPV-16 diagnosis.**

***Index Terms*—Au nanoparticles, diagnosis, human papillomavirus (HPV).**

I. Introduction

More than 400 000 women are diagnosed with cervical cancer in the world each year [1]. Studies showed that dozens of human papillomavirus (HPV) serotypes (including types 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 68, 73, and 82) are called "high-risk" types, because the infection of those HPV types are linked with cervical cancer [2]. Furthermore, high-risk HPV type 16 (HPV-16) and type 18 (HPV-18) are together responsible for over 70% of cervical cancer cases. It was reported that HPV-16 causes 41%–54% of cervical cancers and accounts for an even greater majority of HPV-induced vaginal cancers [3], [4].

Cervical cancer screening is currently based on the Papanicolaou (Pap) smear, which has had a vast impact on the reduction in the incidence of this disease in developed countries. However, the sensitivities of cytological tests vary greatly according to the experience of the cytologists and the type of quality control in place [5]–[9]. The clinical value of HPV DNA testing has increasingly been recognized. Independent studies have shown that HPV DNA detection, when it is used as a primary screening method, has a higher sensitivity and a higher negative predictive value for the detection of preinvasive disease than conventional Pap smear or liquid-based cytology methods [10]–[12]. Constant progress in HPV subtyping based on polymerase chain reaction (PCR) methods has been made over the past few years. The use of degenerate and/or consensus primers offers the advantage of detecting a large spectrum of HPV types by a single PCR [13]–[15]. However, they may be less efficient in detecting specific HPV types, leading to some under detection, particularly in cases of multiple infections.

Most DNA detection methods are based on the DNA hybridization between single-stranded DNA (ssDNA) probes and their complementary DNA targets [16]–[18]. Recently, oligonucleotide probes have also been capped onto Au nanoparticles (AuNPs), called oligonucleotide-functionalized AuNPs or AuNP probes, after which the AuNP probes were used to

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hybridize to target sequences [19], [20]. The possibility of linking the AuNPs through hybridization with target DNA led to the formation of AuNPs aggregates in the micrometer size range. This phenomenon induces a color change from red for free particles in solution to blue (or purple) for aggregated ones, providing the basis of a direct colorimetric detection of the target DNA in detection solution [21]–[23]. In addition, aggregation of the AuNPs, which may be induced by the addition of salt, would change the visible color of a colloidal solution from red into purple; aggregation of the AuNPs shifts the absorption peak toward a longer wavelength [24].

In this study, we developed a reliable optical detection method of specific AuNP probe assaying for the purposes of discriminating high-risk HPV-16 and HPV-18 sequences. Asymmetric PCR performed with universal primers could theoretically amplify the target sequence from several HPV subtypes. The PCR products were used to react with HPV-16- or HPV-18-specific AuNP probes, such that HPV-16 and HPV-18 sequences could be visibly detected by a color change through controlling DNA hybridization conditions.

TABLE I
SEQUENCES OF PROBE, TARGET, AND PRIMER OLIGONUCLEOTIDES DESIGNED AGAINST THE SEQUENCES WITHIN THE L1 GENE OF HPVS

Specific probe oligonucleotides for HPV-16 and HPV-18 detection	
HPV16G1	5' HS-$(CH_2)_6$ACTGAAAATGCTAGTGCTTATGCAGCAAAT-3'
HPV18G1	5' HS-$(CH_2)_6$ACTGAAAGTTCCCATGCCGCCACGTCTAAT-3'
HPV16G2	5'-GTGTGGATAATAGAGAATGTATATCTATGG- $(CH_2)_6$-SH 3'
HPV18G2	5'-CTGAGGACGTTAGGGACAATGTGTCTGTAG-$(CH_2)_6$-SH 3'
Target sequences of HPV-16 and HPV-18	
T64-16	5'-CCATAGATATACATTCTCTATTATCCACACCTGCATTTGCTGCATAA GCACTAGCATTTTCTGT-3'
T64-18	5'-CTACAGACACATTGTCCCTAACGTCCTCAGAAACATTAGACGTG GCGGCATGGGAACTTTCAGT-3'
Universal PCR primers	
HPVU-F	5'-AATAAATTGGATGACACTGA-3'
HPVU-R	5'-CAACTGTGTTTGTTTATAATC-3'

II. MATERIALS AND METHODS

A. Oligonucleotides and Chemicals

All oligonucleotides were designed using Primer Express software (Applied Biosystems, Foster, CA) and were also synthesized by Applied Biosystems. They are listed in Table I, which includes the oligonucleotides of probes, targets and PCR primers. For the sequence detection within the HPV-16 L1 gene (Genbank DQ469930) and HPV-18 L1 gene (Genbank X05015), the specific oligonucleotide probes (30-mer) were synthesized, which include the probes of HPV16G1, HPV16G2, HPV18G1, and HPV 18G2. The probes of HPV16G1 and HPV16G2 are a pair for specifically detecting HPV-16, and HPV18G1 and HPV18G2 are a probe pair for HPV-18 detection. These probes were modified with thiol-linkered tag $[\mathrm{HS}\text{-}(\mathrm{CH}_2)_6]$ at the $5'$ or $3'$ terminus of the oligonucleotides for capping to AuNPs. The target oligonucleotides in length of 64-mer, T16–64 and T18–64, were synthesized, and the partial sequences of these target oligonucleotides are complementary to their probe sequences, which were capped onto AuNPs for DNA sandwich hybridization (Table I). For amplifying the target DNA sequences from real samples of clinical specimens by PCR, the universal primer pair, HPVU-F and HPVU-R, specific for amplifying the DNA fragment within HPV L1 gene was used.

All other chemicals such as sodium chloride (NaCl), sodium citrate ($\mathrm{C_6H_5Na_3O_7}$), chloroauric acid ($\mathrm{HAuCl_4 \cdot 3H_2O}$), and sodium phosphate ($\mathrm{Na_2HPO_4}$) were purchased form Sigma-Aldrich (St. Louis, MO).

B. Patient Samples and Cell Lines

For the patient studies described here, the population consisted of 70 women who were diagnosed as having cervical intraepithelial neoplasia (CIN), defined as CIN-1, CIN-2, or CIN-3, at the Chang Bing Show Chwan Memorial Hospital, Taiwan, during 2007. Lesion biopsies were obtained from all cases, and all histologic sections were examined by experienced pathologists. Exfoliated ectocervical and endocervical cell specimens were collected from all participants using an Accelon Biosampler (Medscand, Hollywood, FL) to allow for cytological and HPV-DNA analyses.

HPV-positive cells, CC7T/VGH (BCRC 60195) and HeLa (ATCC CCL-2) cell lines were purchased from Bioresources Collection and Research Center (Food Industry Research and Development Institute, Hsinchu, Taiwan). CC7T/VGH and HeLa cells were used in this study as HPV-16- and HPV-18 positive controls because the genomes of these cells are known to contain HPV-16 and HPV-18 sequences, respectively [25], [26].

C. Synthesis and Size Characterization of AuNPs

The 13-nm AuNPs were prepared by the sodium citrate reduction method [27], [28]. An amount of 22.5 mL of 1 mM chloroauric acid solution was heated to 130 °C, and when the solution was vigorously mixed, 2.5 mL of 38.8 mM sodium citrate was added as quickly as possible into the solution to keep the reduction time at 130 °C for 5 min, after which the solution was left to cool at room temperature. Diameters of the prepared AuNPs were measured by a scanning electron microscope (SEM) (Quanta 400 FEG scan electrical microscopy; FEI Company, Hillsboro, OR), and absorption spectra of the AuNPs were measured by a spectrophotometer (SpectraMax 190; Molecular Devices Corporation, Sunnyvale, CA).

D. Preparation of AuNP Probes

Solutions of the AuNP probes were prepared according to previously reported procedures with minor modifications [29], [30]. Briefly, 2 nmol of thiolated oligonucleotide probes were mixed with the 13-nm AuNPs (6 nM) solution at 37 °C for 24 h. The solution was then changed to 0.05 M NaCl (in 10 mM $\mathrm{NaH_2PO_4}$ phosphate buffer [PB], pH 7.0) and was kept at 37 °C for 6 h; then the concentration of NaCl was increased gradually to be 0.1, 0.2, and 0.3 M NaCl step by step, with solution being kept at each step for 6 h.

The excess probes were removed from the modified AuNPs as follows. The AuNP probes solution was centrifuged for 20 min at 12 000 $\times$ g, the supernatant was decanted, and the residue was dispersed in 0.3 M NaCl solution (in PB, pH 7.0). The procedures were repeated again, and a final reddish residue was dispersed in 0.3 M NaCl solution and filtered through 0.22 μm pore size membrane. The concentration of AuNP probes solution was determined by absorbance at 520 nm. Finally, the AuNP probes solution was stored in 0.3 M NaCl at 4 °C for further use.

E. Procedure of DNA Sandwich Hybridization

The principle of the AuNP probes used in the operation of DNA sandwich hybridization is depicted in Scheme 1. Equal volumes of the AuNP probes pair for HPV-16 or HPV-18 were mixed, with the final volume containing 100 μL of AuNP probes solution. Next, 1 μL of 10 μM synthesized oligonucleotide targets or PCR products were added into the solution and incubated at 60 °C for HPV-16 or 50 °C for HPV-18 detection (the temperature is below the theoretical melting temperature [Tm] by 5 °C). Fifteen minutes after hybridization, 100 μL NaCl solution (in 10 mM Na_2HPO_4, pH 7.4) was added to the solution, making the salt concentration in the reaction mixture 1.0 M NaCl. The reaction solution was then iced for 5 min and kept at 4 °C to await detection. An absorption spectra (400–700 nm) or an absorption value at 520 nm of the reaction solutions was determined by the SpectraMax 190 spectrophotometer. A sample of the reaction solutions was also checked by electrophoresis on a 3% agarose gel. The AuNPs could be directly observed on the agarose gel. Furthermore, when the agarose gel was stained with ethidium bromide, the hybrid DNA of AuNP probes and target sequences was then observed.

F. Extraction of HPV Genomic DNA

Total genomic DNA was extracted from CC7T/VGH, HeLa cells, and specimens isolated from patients using the Wizard Genomic DNA Purification Kit (Promega, Madison, WI) according to the manufacturer's protocol. For each preparation, a total of 50 μL DNA was eluted from the DNA purified column and examined by 0.8% agarose gel electrophoresis. The concentration of extracted DNA was measured by the SpectraMax 190 spectrophotometer at 260 nm.

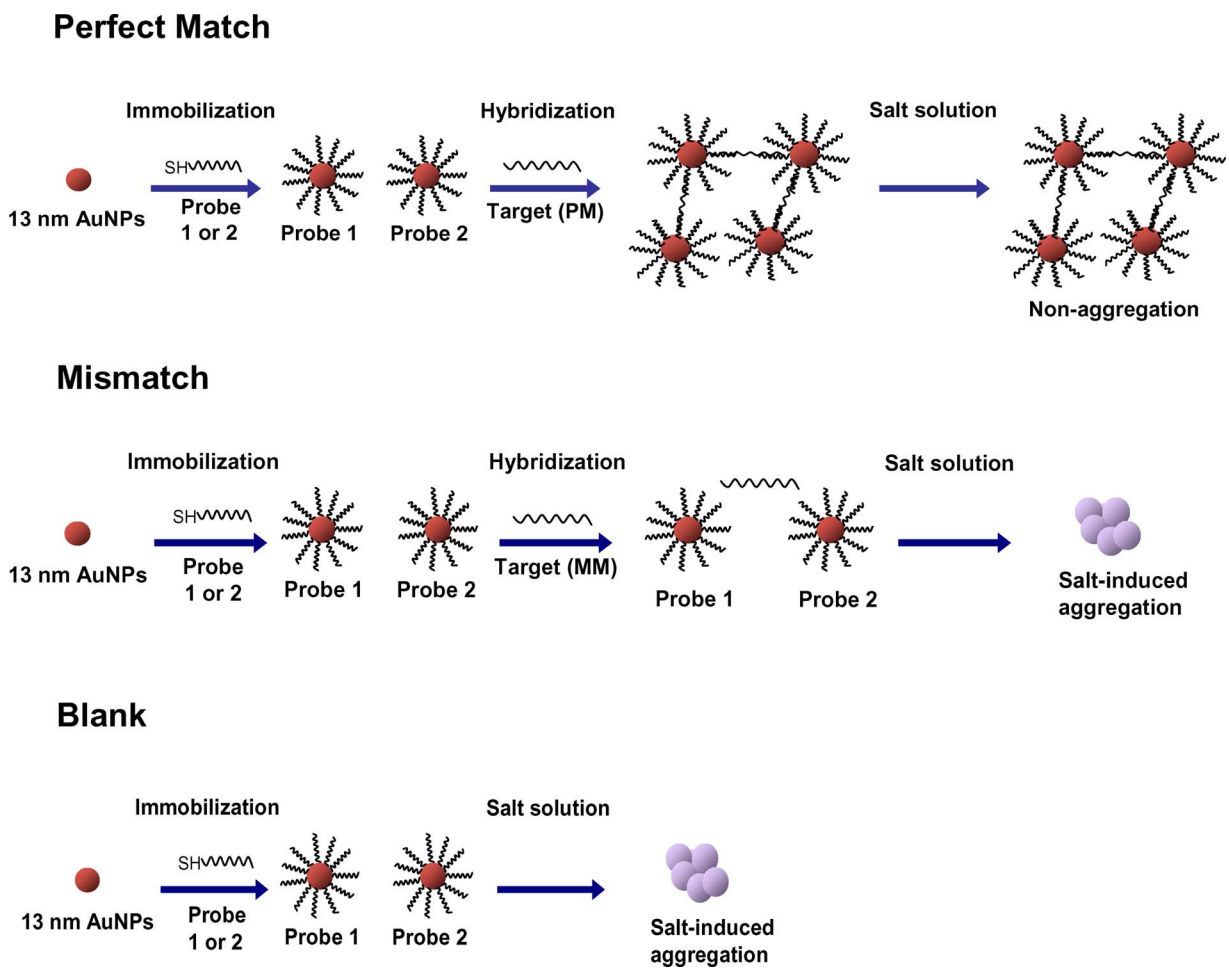
G. Asymmetric PCR

All target DNA fragments of HPV from cervical specimens and cell lines were amplified by the method of asymmetric PCR with a universal primer pair. The primers, HPVU-F and HPVU-R, are nearly adjacent to both sides of the target L1 region of HPV, e.g., the universal primer (forward/reverse) located at nt position 370/468 within the HPV-16 L1 region, and at nt position 553/651 within the HPV-18 L1 region. Moreover, the universal primer pair could be used to amplify any one of at least 45 HPV genotypes (including HPV-2a, 6, 8, 10, 11, 13, 16, 17, 18, 20, 21, 24, 26, 27, 30, 31, 33, 34, 35, 37, 39, 42, 43, 44, 45, 51, 52, 53, 54, 55, 56, 58, 59, 61, 66, 68, 69, 70, 71, 74, 81, 82, 83, 84, 86, and 89) in theory [31]. The universal primer pair was used to amplify the target DNA fragments of approximately 99 bp in size from the template DNA extracted from the mucous specimen, HeLa, or CC7T/VGH cells.

PCR reactions contained 2 μL of genomic DNA (around 50 ng), 1 μL of 10 μM HPVU-F and 1 μL of 1 μM HPVU-R primers, 5 μL of 10$\times$ PCR buffer (100 mM Tris HCl [pH 9.0], 15 mM $MgCl_2$, 500 mM KCl, 1% [v/v] Triton X-100, and 0.1% [w/v] gelatin), 2 μL of 10 mM deoxynucleoside triphosphate (dNTP) mixture, 1 μL of 0.5 U Super Taq DNA polymerase (HT Biotechnology Ltd., Cambridge, England), and 38 μL distilled water in a total volume of 50 μL. PCR was performed in a PTC-100 thermal cycler (MJ Research, Inc., Waltham, MA), and PCR conditions were as follows: initial denaturation at 94 °C for 5 min, followed by 42 cycles of denaturation at 94 °C for 30 s, annealing at 40 °C for 30 s, and elongation at 68 °C for 30 s, with a final extension at 68 °C for 3 min. The asymmetric PCR products were purified in a total volume of 50 μL TE buffer using Wizard PCR Preps DNA Purification System (Promega) according to the manufacturer's protocol and checked by electrophoresis on an urea/acrylamide gel (7 M of urea and 40% acrylamide/bis-acylamide) for specifying ssDNA detection [32]. After electrophoresis, the acrylamide gel was stained with 0.02% methylene blue. The concentration of amplified DNA was measured using a SpectraMax 190 spectrophotometer at 260 nm.

H. TaqMan Real-Time PCR Assay

The TaqMan real-time PCR assay for HPV-16 diagnosis was performed by the procedure reported by Davidson *et al.* [33] with slight modifications. All patient samples were detected using the method of TaqMan real-time PCR assay by an ABI Prism 7700 sequence detection system (Applied Biosystems). Amplification of specific PCR products was detected using dual-fluorescent nonextendable probes labeled with 6-carboxyfluorescein (FAM) at the 5′ end and with 6-carboxytetramethylrhodamine (TAMRA) at the 3′ end. The characterizations of the specific HPV16 primer pair and probe are as follows: forward primer: HPV 16 L1–278F (5′-CAG ATA CAC AGC GGC TGG TTT-3′, nt position 278–299 of HPV 16 L1 ORF), reverse primer: HPV 16 L1–417R (5′-TGC ATT TGC TGC ATA AGC ACT A-3′, nt position 396–417 of HPV 16 L1 ORF), and probe: HPV 16 L1–302pr (5′-FAM-TGA CCA CGA CCT ACC TCA ACA CCT ACA CAG G-TAMRA-3′, nt position 302–333 of the HPV 16 L1 ORF) [33]. The TaqMan real-time PCR was performed in a total reaction volume of 50 μL containing 1$\times$ TaqMan buffer (Applied Biosystems), 300 nM forward and reverse primers, 100 nM dual-labeled fluorogenic internal probe, 1.25 U Ampli*Taq* Gold DNA polymerase (Applied Biosystems), and 50 ng total DNA from each patient as a template. The reaction was performed in MicroAmp optical 96-well plates covered with MicroAmp optical caps (Applied Biosystems). PCR was initiated by a pre-incubation step at 50 °C for 2 min followed by an incubation step at 95 °C for 10 min. Subsequently, 40 two-step PCR cycles with a denaturation step at 95 °C for 15 s and an annealing step at 60 °C for 1 min



Scheme 1. Schematic presentation of sequences sandwich hybridization with oligonucleotide-functionalized AuNPs. A pair of specific oligonucleotide probes was capped onto 13-nm AuNPs to generate AuNP probe 1 and AuNP probe 2. The target sequences were then used to sandwich hybridized with the AuNP probes set at certain temperature and NaCl concentration. After DNA hybridization, the NaCl concentration of reaction solution was increased and the resulting AuNP probes aggregated if the AuNP probes were not linked to their complementary (i.e., perfect match) target sequences. The results can be easily read by the naked eye or determined by a photospectrometer at 520 nm, because the reaction solution with hybridized AuNP probes and target sequences present a red color; otherwise, a large amount of aggregated AuNP probes in the blank (no target sequences applied) or a mismatch (the AuNP probes failure to hybridize with the noncomplementary target sequences) display a light purple color in a transparent 96-well petri dish.

were completed. Furthermore, data from the ABI Prism 7000 sequence detection instrument were analyzed with Sequence Detector software version 2.0.

III. Results

A schematic presentation of the sequence sandwich hybridization with oligonucleotide-functionalized AuNPs is illustrated in Scheme 1. The 3′ and 5′ thiol-modified DNA probes were separately and covalently immobilized (capped) onto the surface of 13-nm AuNPs to generate a pair of oligonucleotide-functionalized AuNPs, named AuNP probes. In this study, pairs of HPV16G1/16G2 and HPV18G1/18G2 AuNP probes were generated and used to detect HPV-16 and HPV-18, respectively. The DNA sandwich hybridization was performed in the reaction with 0.3 M NaCl buffer at an appropriate temperature, which is dependent on the Tm between AuNP probes and target sequences. After DNA hybridization, the NaCl concentration of the reaction solution was increased. Following an increase in salt concentration and then quick cooling of the reaction solution, a blank and the hybridization with mismatch sequences showed extensive AuNP aggregation, noticeable by the purple color of the respective solutions. In contrast, the hybridization with the complementary (i.e., perfect match) sequences did not show the effect, and the solution retained the initial red color. Full hybridization of the probe sequences capped on AuNPs to their complementary target sequences appears to stabilize the AuNPs in solution and to avoid AuNP aggregation. Color change upon aggregation of AuNP probes is corroborated by visible spectra, where an intense plasmon resonance band appears at 600–650 nm (purple in color observed by naked eye), with a concomitant decrease in the intensity of the original plasmon resonance at 520 nm (red).

A. Temperature Effect on DNA Hybridization

To test temperature effect on DNA hybridization, the pair of AuNP HPV16G1/16G2 was used to hybridize with the synthesized target sequence T64-16 at 20 °C, 30 °C, 40 °C, 50 °C, 60 °C, and 70 °C. The absorbent value at 520 nm decreased,

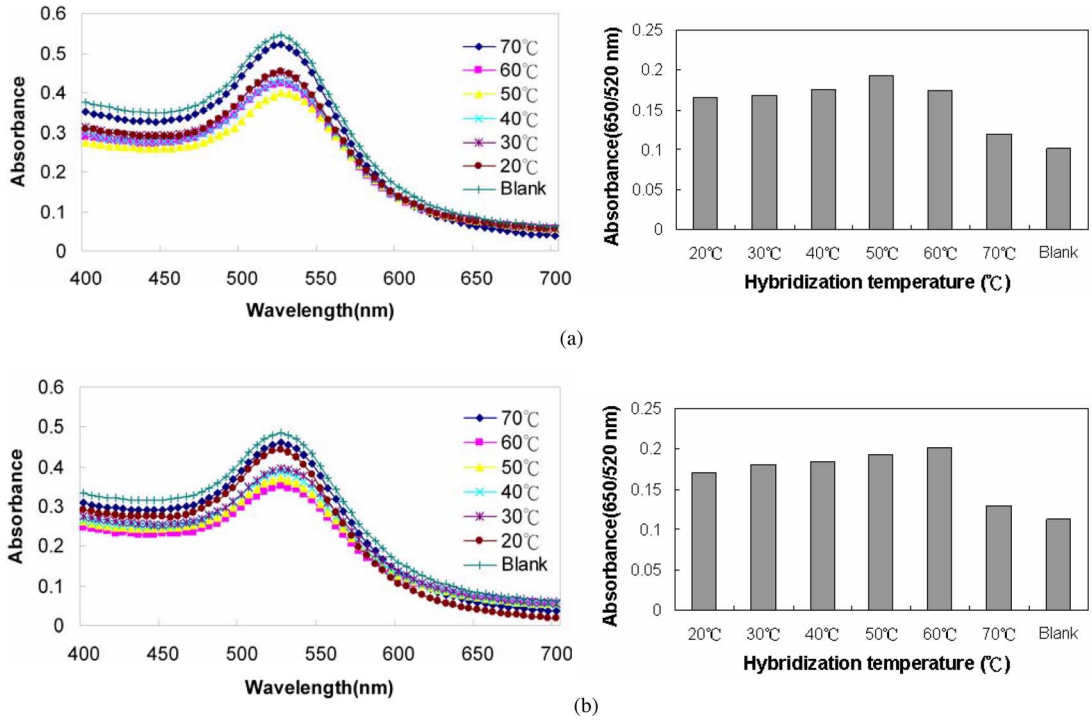


Fig. 1. Effect of temperature on the absorption spectrum of specific AuNP probes hybridized with synthesized target oligonucleotides. Representing the absorption spectra (from 400 to 700 nm) patterns of 1 μM HPV16G1/G2 oligonucleotide-functionalized AuNPs hybridized with (a) 1 μM synthesized target oligonucleotides T64-16 (for HPV-16 diagnosis) and (b) 1 μM HPV18G1/G2 oligonucleotide-functionalized AuNPs hybridized with 1 μM synthesized target oligonucleotides T64-18 (for HPV-18 diagnosis) at the temperature of 20 °C, 30 °C, 40 °C, 50 °C, 60 °C, and 70 °C are shown. The 650/520 nm ratios when the HPV16G1/G2 and HPV18G1/G2 oligonucleotide-functionalized AuNPs were used to hybridize the T64-16- and T64-18-synthesized target oligonucleotides are shown in panels (a) and (b), respectively. Bars indicate the mean ± S.E. from three independent determinations.

and the 650/520 nm ratio increased, when the target sequences hybridized to the probe sequences, which were capped on the AuNPs, because most of the AuNPs were linked by target sequences to form trimer or tetramer structures [34], [35]. Therefore, as the results in Fig. 1(a) demonstrate for detection of the HPV-16 target sequence T64-16, the DNA hybridization at 50 °C, which is closest to Tm (53 °C), caused the lowest absorbent value at 520 nm and the highest 650/520 nm ratio compared to those detected at the other temperatures. A similar phenomenon also existed in the detection using AuNPs-HPV18G1/18G2 hybridized to the synthesized HPV-18 target sequence T64-18. The DNA hybridization at 60 °C, which is closet to Tm (65 °C), had the lowest absorbance at 520 nm and the highest 650/520 nm ratio [Fig. 1(b)].

Temperature did affect the specificity of DNA hybridization between AuNP probes and target sequences. As AuNP probes hybridized with their target sequences at a temperature far lower than their Tm, the possible forms of hybridization varied, i.e., there were some undesired formations, including hairpins and nonspecific alignments [36]. These undesired formations prevented the hybridization form for which we had specifically designed. However, when hybridization progressed at a temperature near the Tm, there was a lower absorbance at 520 nm and a higher 650/520 nm ratio, such that fewer undesired formations could increase the possibility of perfect matching between the probe and target sequences. Hybridization at a temperature slightly lower than the Tm resulted in more specific matches.

B. *Salt Effect on Detection*

After DNA hybridization of AuNP probes and target sequences, salt concentration increased from 0.5 to 1.0 M in the reaction mixture, and then rapidly cooled to 4 °C. The color of all three mixture solutions, blank (the reaction mixture with AuNP probes, but without target sequences), PM (with AuNP probes and the perfect match target sequences), and MM (with AuNP probes and the mismatch target sequences), under the 0.5 M NaCl condition were similar; however, color changes were different under 0.9 and 1 M NaCl conditions due to the massive aggregations of AuNPs that remained in the precipitate and the fewer AuNPs that were suspended in the solution. In the blank, the color of the mixture gradually changed from red to light purple when the salt concentration was increased from 0.5 to 1.0 M in the reaction mixtures. However, in the PM group, the color of the mixture maintained its original red color. In the MM group, it displayed an immediate color change from red to purple (Fig. 2(a) for HPV-16 detection and Fig. 2(b) for HPV-18 detection). These results show that there were distinct differences in color between the hybridizations of PM and MM under the conditions of 1.0 M salt buffering.

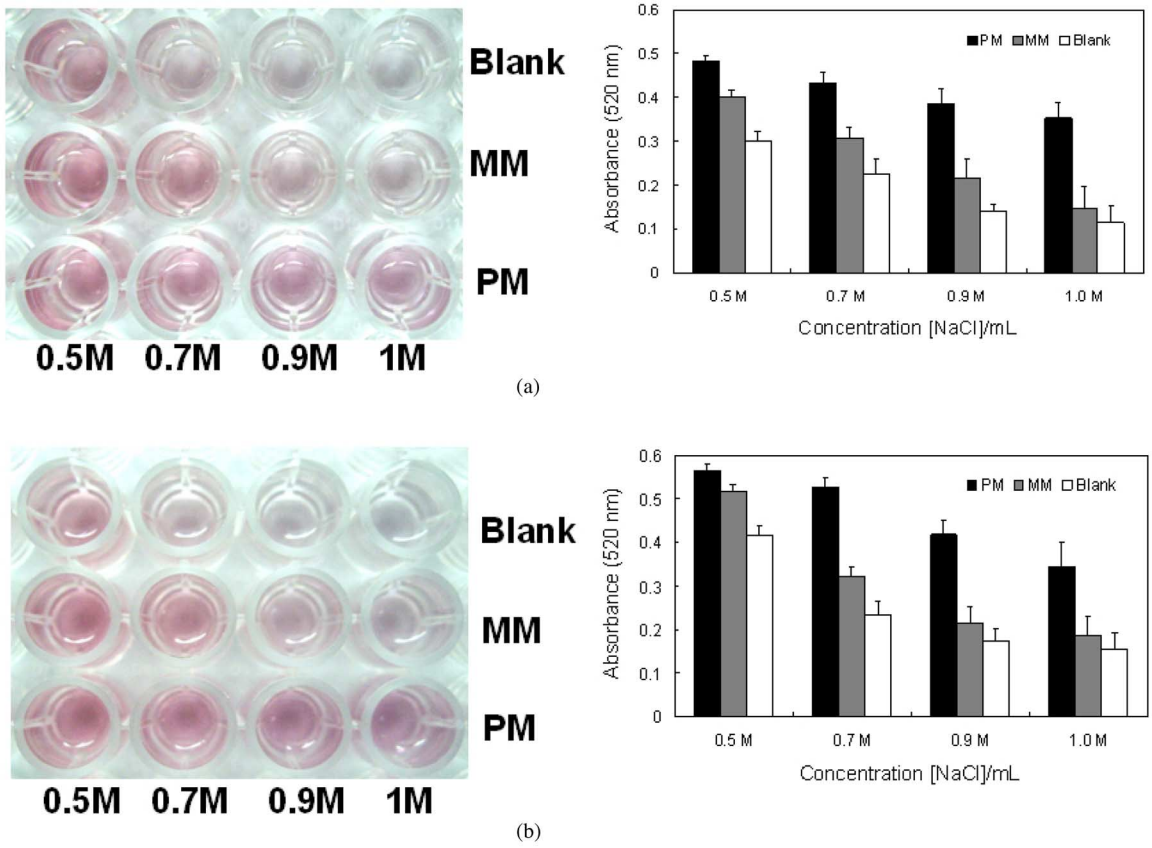


Fig. 2. Effect of buffering salt concentration on the absorption spectrum of the specific AuNP probes hybridized with the synthesized target oligonucleotides. Representative photographs show that (a) HPV16G1/G2 oligonucleotide-functionalized AuNPs hybridized with the synthesized target oligonucleotides T64-16 (perfect match, PM), T64-18 (mismatch, MM), and without oligonucleotide sequences (blank) and (b) HPV18G1/G2 oligonucleotide-functionalized AuNPs hybridized with the synthesized target oligonucleotides T64-18 (PM), T64-16 (MM), and without oligonucleotide sequences (blank) under the condition with buffer of 0.5, 0.7, 0.9, and 1.0 M NaCl in a 96-well petri dish. The absorption values at 520 nm of either HPV16G1/G2 or HPV18G1/G2 oligonucleotide-functionalized AuNPs used to hybridize the (a) T64-16- and (b) T64-18-synthesized target oligonucleotides and without oligonucleotide sequences under the condition with 0.5, 0.7, 0.9, and 1.0 M NaCl buffer were detected. Bars indicate the mean $\pm$ S.E. from three independent determinations. The reactions of HPV16G1/G2 oligonucleotide-functionalized AuNPs hybridized with synthesized target oligonucleotides T64-16 (PM), T64-18 (MM), and without oligonucleotide sequences (blank) under the condition of 1.0 NaCl buffer were observed by SEM. The SEM photographs show AuNP aggregation in the treatments of (c) blank and (d) MM, and (e) AuNP dispersal as well as two to four AuNPs linked together in the PM treatment.

UV-vis spectrophotometry was used in order to obtain a more detailed picture of the amplitude of color change observed by the naked eye. The results of measurements at 520 nm for all three reaction mixtures, blank, MM, and PM, were consistent to those observations made by the naked eye [Fig. 2(a) and (b)].

To characterize the stability of AuNP probes, SEM was used to observe AuNP distribution in the reaction mixture. In the 1.0 M salt buffer, AuNPs capped with specific probes without reacting with the target oligonucleotides showed significant AuNP precipitation and aggregation [Fig. 2(c)]. In the same salt condition, the AuNP probes that hybridized with their MM target oligonucleotides also showed that AuNP probe aggregation occurred [Fig. 2(d)]. However, the AuNP probes that hybridized with their PM target oligonucleotides demonstrated AuNP probe dispersal with two to three AuNPs being linked [Fig. 2(e)]. As such, the AuNP probes linked with the PM

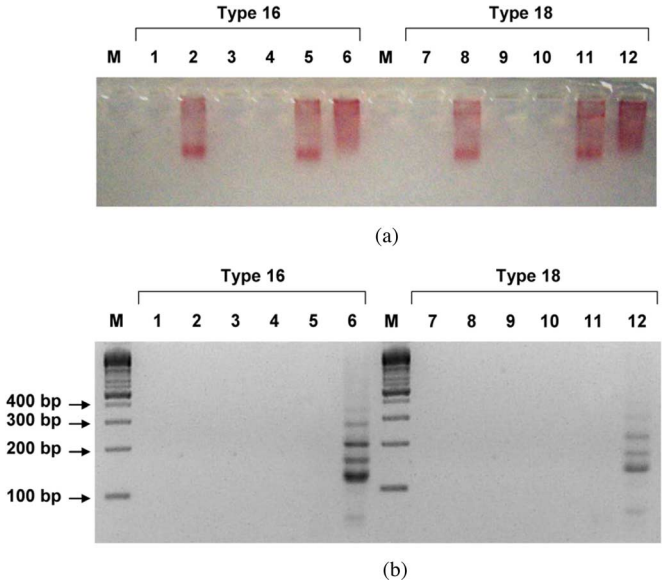


Fig. 3. Photographs of agarose gel electrophoresis with the AuNPs or specific AuNP probes hybridized with the synthesized target oligonucleotides. The hybridizations were performed under buffering 0.3 M NaCl at 50 °C for HPV-16 and at 60 °C for HPV-18 detection. (a) Visible 3% agarose gel was used to identify the AuNPs existing in the hybridization solution and (b) gel stained with ethidium bromide for confirming the hybrid DNA of AuNP probes and target sequences. M indicates the DNA marker and the molecular weight of 100, 200, 300, and 400 bp indicated; lanes 1 and 7 indicate the reactions with only AuNPs; lanes 2 and 8, HPV16G1/G2- and HPV18G1/G2-capped AuNP probes, respectively; lanes 3 and 10, T64-16 target oligonucleotides; lanes 4 and 9, T64-18 target oligonucleotides; lane 5, HPV18G1/G2-capped AuNP probes hybridized with T64-16 target oligonucleotides; lane 6, HPV16G1/G2-capped AuNP probes hybridized with T64-16 target oligonucleotides; lane 11, HPV16G1/G2-capped AuNP probes hybridized with T64-18 target oligonucleotides; lane 12, HPV18G1/G2-capped AuNP probes hybridized with T64-18 target oligonucleotides. (a) In the lanes 2, 5, 6, 8, 11, and 12, the AuNPs as red color on the gel were visible. (b) In the lanes 6 and 12, hybrid DNA for AuNP probes and target sequences were observed by ethidium bromide staining.

target oligonucleotides supported the structure form and negative charge could prohibit AuNP probes aggregation by electrical charge attraction [34], [35].

C. DNA Hybridization Assay by Electrophoresis

Agarose gel electrophoresis was used to examine DNA hybridization between different sequences of the AuNP probes, which were used to hybridize different sequences of target oligonucleotides. As shown in Fig. 3, the AuNPs in the agarose gel could be directly observed in red by the naked eye [Fig. 3(a)], and a DNA hybrid of the AuNP probes and their complementary target sequences was observed in ethidium bromide-stained agarose gels [Fig. 3(b)]. In lanes 2, 5, 8, and 11 in Fig 3(a), the main red band could be observed because there were AuNP probes present (HPV16G1/G2- or HPV18G1/G2-capped AuNPs); in lanes 6 and 12, a red smear signal indicated that the HPV16G1/G2- and HPV18G1/G2-capped AuNPs hybridized with the target oligonucleotides of T64-16 or T64-18, respectively. In a parallel electrophoretic gel stained with ethidium bromide, the results showed that only lanes 6 and 12 in Fig. 3(b) resulted in a stained signal. This indicates that the DNA hybrid formed when the AuNP probes hybridized with their complementary target sequences.

The AuNP probes that hybridized with their complementary target sequences resulting in different degrees of linkage caused smears on the electrophoresis gel, as shown in lanes 6 and 12 in Fig. 3(a). Moreover, in lanes 6 and 12 in Fig. 3(b), the DNA fragments resulted in units of multiple size. Theoretically, the sizes of the DNA fragments should be 64 bp multiples, because the DNA hybrid of probes and target sequences are multiples of 64 bp. However, hybridized double-stranded DNA with AuNPs could yield bands slightly larger than 64 bp or multiples of this fragment size.

D. Asymmetric PCR for Patient Samples

To confirm the practicability of this method for clinical testing, we carried out a series of experiments consisting of DNA extraction from human tumor cell lines and patient specimens, PCR amplification of the HPV L1 gene fragment, and optical detection by specific AuNP probes (AuNP HPV16G1/G2 and AuNP HPV18G1/G2). The universal primer pair (Table I) was designed and possibly used to amplify any one of the 45 HPV subtypes. In following the PCR experiments, the universal primer pair was used to amplify the target DNA fragments in size of about 100 bp, using total DNA extracted from a patient's mucous specimen, HeLa cells, or CC7T/VGH cells as templates.

In producing single-stranded target sequences from a sample, asymmetric PCR was utilized to amplify the desired L1 region of the HPV genome from clinical specimens. The asymmetric PCR-amplified ssDNA could not be observed on an agarose gel [Fig. 4(a)]. Therefore, a urea/acrylamide gel was used to demonstrate the asymmetric PCR products in Fig. 4(b). The PCR-amplified ssDNA (30 419 g/mol) attributed to the bands shown on the urea/acrylamide gel was stained with methylene blue, such that the ssDNA would hybridize with the bands specific and complementary AuNP probes.

E. Detection Limit of the Optical Detection Method

The synthesized HPV-16 target oligonucleotides T64-16 from 1.4×10^{0} to $1.4 \times 10^{-5}\,\mu$M at an interval of tenfold serial dilution were prepared for the AuNP probes sandwich hybridization in order to evaluate the detection limit and sensitivity of the measurement of optical absorbance at 520 nm (Fig. 5). The values between the target sequence concentration and absorbance (520 nm) were highly correlated, and a linear relationship was demonstrated ($R^2 = 0.972$). The detection limit was as low as $1.4 \times 10^{-4}\,\mu$M of the target sequences in the detection when the threshold for the positive detection was set as the mean + 3S.E. of the blank.

F. Clinical Specimens Assay

For each clinical specimen assay, approximately 200 nM of the targeting DNA produced by the asymmetric PCR was applied and mixed with 90 μL of HPV16G1/G2- or HPV18G1/G2-functionalized AuNPs.

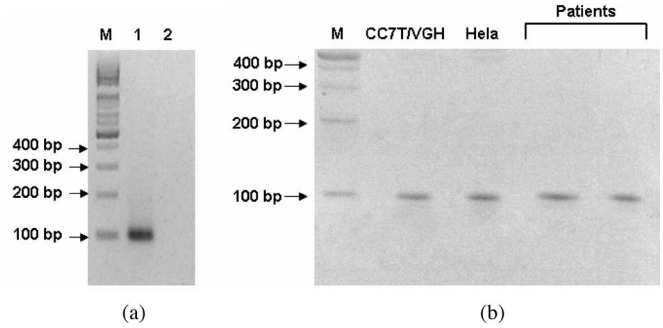


Fig. 4. Photographs of agarose gel and urea/acrylamide gel electrophoresis with asymmetric PCR products. The asymmetric PCR products amplified from total DNA specimens with universal primers, HPVU-F and HPVU-R, were assayed on (a) agarose gel with ethidium bromide staining and (b) urea/acrylamide gel with methylene blue staining for nucleic acids. The ssDNA produced by the asymmetric PCR; therefore, the band of PCR products with exact molecular mass was clearly observed on the urea/acrylamide gel but not in the agarose gel. In panel (a), M indicates the DNA marker and the molecular weight of 100, 200, 300, and 400 bp indicated; symbols 1 and 2 indicate the DNA products from the PCR and asymmetric PCR, respectively. In panel (b), CC7T/VGH and HeLa were the positive controls for HPV-16 and HPV-18 detection, respectively; patients indicate the clinical samples.

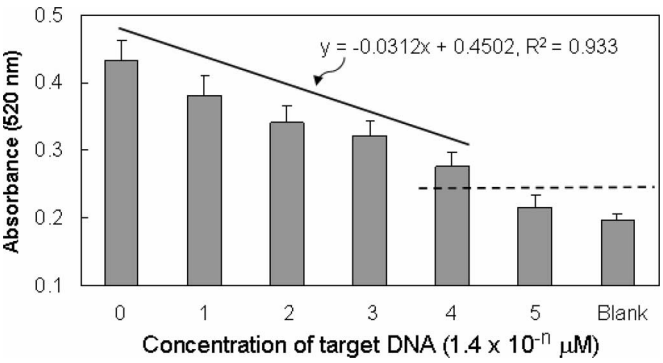


Fig. 5. Detection limit for HPV-16 target sequences detected by the optical detection method using oligonucleotide-functionalized AuNPs. In the detection, synthesized HPV-16 target oligonucleotides T64-16 were detected using the AuNPs modified with HPV-16-specific oligonucleotides (i.e., HPV-16-specific AuNP probes). The target oligonucleotides, from 1.4×10^{0} to $1.4 \times 10^{-5}\,\mu$M at an interval of tenfold serial dilution, were prepared for the AuNP probes sandwich hybridization to evaluate the detection limit and range by the measurement of optical absorbance at 520 nm. The detection without target sequences added was also performed as a blank. A linear relationship was found between the absorbance values (520 nm) versus target sequences concentration from 1.4×10^{0} to $1.4 \times 10^{-4}\,\mu$M. The threshold for the positive detection was set as the mean + 3S.E. of the blank; therefore, the detection limit was determined as $1.4 \times 10^{-4}\,\mu$M of the target oligonucleotides. Each value was derived from three independent detections and error bars indicated S.E.

CCT/CGH and HeLa cells were employed to demonstrate that such a procedure could distinguish HPV-16 and HPV-18, such that CCT/CGH was used for HPV-16 and HeLa was used for HPV-18. Fig. 6 shows that 70 clinical specimens from patients with invasive cervical cancer were tested by the AuNP probes sandwich hybridization method. There were 33 patients whose sample was infected with HPV-16, 16 patients with HPV-18, 5 patients with both HPV-16 and HPV-18 infection, and 16 patients with neither of the two subtypes.

To test the accuracy of our developing optical detection method by specific AuNP probes, a well-known method, the TaqMan real-time PCR assay, was established (results not shown) and used to detect HPV-16 infection in clinical samples. Of the 70 clinical specimens assayed, 35 (50%) were identified as positive, and 30 (43%) were identified as negative, by both the optical detection method (oligonucleotide-functionalized AuNPs) and TaqMan real-time PCR assay. Three specimens were positive only by the optical detection method, and two specimens were positive only by TaqMan real-time PCR assay (Table II). The sensitivity and specificity of the optical detection method compared to the TaqMan real-time PCR assay were 95% and 90%, respectively, for HPV-16 diagnosis.

IV. Discussion

To our knowledge, this is the first investigation to use AuNP probes to perform HPV diagnosis by an optical detection method of DNA sandwich hybridization. This method of optical detection or colorimetric assay was developed for distinguishing HPV-16 and HPV-18 based on specific probe-functionalized AuNPs. The method is simple, fast, and economical for DNA hybridization detection. In this study, controlling temperature and salt conditions was performed in order to optimize the specificity of DNA hybridization and optical differences.

In the detection of HPV, stability of AuNP probes is a crucial factor. The prepared AuNP probes solution exhibited a substantially high level of stability [37]. Increased stability of DNA-modified AuNPs is due to higher oligonucleotide surface coverage, which leads to greater steric and electrostatic protection [38]. The AuNP probes sandwich hybridized with their complementary target sequences were much more stable than those of AuNP probes hybridized with the mismatch target sequences when the reaction was exposed to 1.0 M NaCl, a buffer with higher salt concentration. A high salt concentration causes irreversible AuNP aggregation, which double-stranded DNA cannot prevent, and thus affects the optical properties of AuNPs.

The optical detection method described here is particularly important because this detection system can be minimized or can eliminate the need for using expensive and/or complicated instruments for detection. There are numerous commercially available tests based on the principle of protein–AuNP conjugates, including tests for the detection of infectious microbes and determination of pregnancy. Since 1996, Mirkin and coworkers have reported a novel and useful method, i.e., the DNA sandwich hybridization assay, using oligonucleotide–AuNP conjugates (i.e., AuNP probes) [22], [37], [38]. The method for DNA detection is based on AuNP probes hybridizing with their target sequences, and the detection result is presented as a concomitant red-to-purple color change depending on whether the AuNPs aggregate or not. However, the technology of AuNP probes has not been translated to clinical applications, even though some reports have demonstrated its practicability [39].

The clinical application of AuNP probes still requires further investigation, including DNA hybridization kinetics, effects on

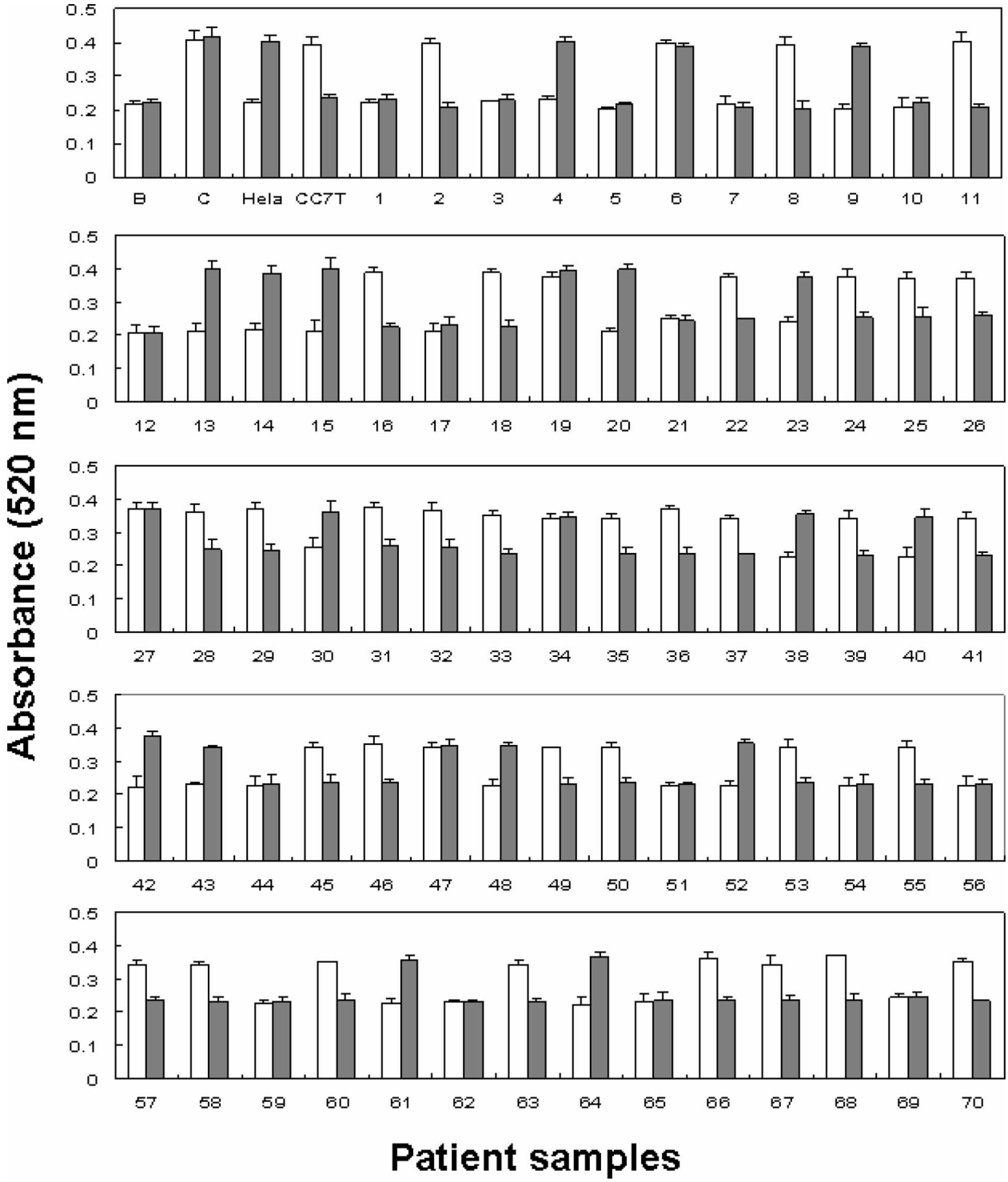


Fig. 6. HPV-16 and HPV-18 diagnosis using the optical detection method of sequences sandwich hybridization with the oligonucleotide-functionalized AuNPs. The asymmetric PCR products were amplified from the 70 cervical specimens and two cell lines with universal primers and applied to the detections by the HPV-16- and HPV-18-specific AuNP probes in a 96-well culture dish and then determined by a spectrophotometer at 520 nm. The result shows that there were 33 specimens showing infection with HPV-16, 16 with HPV-18, 5 with both HPV-16 and HPV-18, and 16 specimens free of HPV-16 and HPV-18 infection. CCT/VGH cells were used in the test as HPV-16 positive control and HeLa cells were as HPV-18 positive control.

DNA hybridization (e.g., ionic concentration and reaction temperature), probe and target sequence characteristics (e.g., percentage of GC content and length of the sequences), etc. This is one of the reasons why using the optical detection method of AuNP probes for clinical applications is strictly limiting. We also understand the concern regarding the validity of viral sequence diagnosis in clinical specimens in the context of contaminating human whole genome DNA, which is unavoidable.

TABLE II
COMPARISON OF OPTICAL DETECTION METHOD VERSUS TAQMAN REAL-TIME PCR ASSAY FOR HPV-16 DIAGNOSIS IN 70 CLINICAL SPECIMENS

HPV-16 diagnosis		TaqMan real-time PCR assay	
		Positive	Negative
Optical detection method (oligonucleotide-functionalized AuNPs)	Positive	35 (True positive, TP)	3 (False positive, FP)
	Negative	2 (False negative, FN)	30 (True negative, TN)
		Sensitivity = [TP/(TP+FN)] x 100% = 95%	Specificity = [TN/(FP+TN)] x 100% = 90%

Based on the results detected by TaqMan real-time PCR, sensitivity and specificity of the optical detection method for HPV-16 diagnosis using the oligonucleotide-functionalized AuNPs (i.e., AuNP-probes) were 95% and 90%, respectively.

Therefore, the sequence specificity of the probe, primer and target and the conditions for DNA sandwich hybridization were strictly tested before the sequences were applied in the optical detection method developed in this study for HPV-16 and HPV-18 diagnosis (data not shown). Therefore, nonspecific detection could be prevented, and a lower detection limit was achieved in this study.

The colloidal gold solution color was very sensitive for measuring differences in the natural (red) and aggregated complex (purple) of AuNPs [40]. Aggregation of the AuNPs, which may be induced by addition of salt, changes the color of the colloidal solution from red into purple, and aggregation of the AuNPs shifts the absorption peak toward a longer wavelength. Accordingly, we tried to develop a model of specific, rapid and reliable AuNP probes assaying for the discrimination of high-risk HPV-16 and HPV-18 sequences. In this study, the functionalized AuNP probes hybridized with their complementary target oligonucleotides at an adequate temperature and could effectively stabilize AuNP probes against salt-induced aggregation. As shown in Figs. 2 and 3, we demonstrated that, when the target sequences existed in the AuNP probes colloidal solution at 50 °C (for HPV-16 detection) or 60 °C (for HPV-18 detection), the target sequences could link AuNP probes together. As shown in the DNA electrophoresis photograph in Fig. 3, even when the salt concentration reached 1.0 M, there were several DNA bands between 50 and 300 bp observed due to different numbers of AuNP probes that were linked by the target sequences.

When determining probe-functionalized AuNPs, the addition of a high-concentration salt buffer (e.g., 1.0 M) followed by cooling to 4 °C rapidly led to a screening effect that was enough to overcome the repulsion interaction between AuNP probes without target sequences. Since the surfaces of AuNPs are covered with ions (such as citrate and chloride ions) and the probes attached to AuNPs provide a negative charge as well, cations (Na^+) in salt solution could neutralize this negative surface charge of AuNPs [41]. Once the electrostatic repulsion between AuNPs decreased, aggregation occurred easily. On the other hand, the decreasing absorption in mismatch with higher salt concentration indicated that an additional cation (Na^+) also balanced the negative surface charge of linked AuNPs. Linked AuNPs could be considered as larger particles, which require more cations to neutralize the negative surface charge of AuNPs [41]. If the concentration of additional salt solution was high enough to neutralize all negative charges on linking AuNPs, there was a sharp decrease in absorption in the reaction. Therefore, an appropriate concentration of additional salt solution was important. A low-temperature procedure was used for rapid aggregation and colorimetric change. Cooling the solution after adding salt solution could lower the free energy of aggregation. As a result, a great deal of precipitation and color change in colloidal solution from red to purple, which appeared in noncomplementary sequence detections and blanks (Fig. 2), allows for operators to easily distinguish between target oligonucleotides with mismatching or complementary sequences.

As mentioned earlier, sandwich hybridized DNA seems to have a protective effect for AuNPs against aggregation, possibly through the electrostatic interactions between the negatively charged phosphate groups on the nucleic acid backbone and the charge in the salt solution [42]. The presence of duplex DNA in solution may act as a "buffer" for increasing the ionic strength, thus stabilizing the AuNPs in the nonaggregated form [43]. The increased stability observed for the complementary target sequences may be explained by this buffering effect, adding to the fact that full sequences of the AuNP probes are hybridized with the complementary target DNA sequences. The resulting duplex DNA prevents salt-induced precipitation. Destabilization of the duplex DNA was derived from the mismatched sequences, and hence the level of AuNP aggregation. These methods make use of this destabilization effect in order to achieve detection of single-base variations, such as single nucleotide polymorphisms (SNPs), and subtype detection of infectious microbes [24].

The approximate time required for specimen processing and assay performance was recorded. Approximately, 30 and 90 min were required for HPV DNA extraction from clinical specimens and PCR for amplifying target HPV DNA fragments, respectively, and only 20 min was required for DNA sandwich hybridization. Accordingly, the total time requirement for the optical detection developed in our study is approximately 2.5 h. Although the time required for specimen processing and PCR is not efficient compared with conventional methods that are performed by an overnight procedure for DNA hybridization, DNA hybridization time in the optical detection method using the AuNP probes is truly rapid when compared to conventional methods (20 min versus 12–16 h) to identify specific DNA sequences.

In summary, we have developed a colorimetric assay for determining HPV-16 and HPV-18 by monitoring the color change of AuNP probes hybridized with their complementary target sequences by increasing the salt concentration of the buffer. In this study, a 1.0 M salt solution was utilized to neutralize the negative charge on AuNPs with mismatching target sequences, but not the AuNPs cross linked with complementary and perfect match sequences. Therefore, mixing the PCR products with mismatched sequence to the AuNP probes resulted in a great deal of

aggregation and changed the color from red to purple rapidly. In contrast, when mixing the PCR products with complementary target sequences to the AuNP probes, the AuNPs maintained their stability against salt-induced aggregation; therefore, the color did not change. While using this method to detect HPV-16 in the 70 clinical specimens from patients with CIN, the detection specificity of the AuNP probes sandwich hybridization reached 95% and 90% compared to those specimens diagnosed by the method of TaqMan real-time PCR. In this study, the optical detection method using AuNP probes is sensitive, label-free, and not requiring expensive equipments, and the detection results can also be read by the naked eye without any optical equipment. These results indicate that the optical detection of HPV-16- and HPV-18-based specific probe-functionalized AuNPs and asymmetric PCR may be useful for the purposes of clinical assays.

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