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A high yield gold nanoparticle-based DNA isolation method for human papillomaviruses genotypes from cervical cancer tissue samples

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ABSTRACT

Gold nanoparticles (AuNPs) are commonly used in biosensors of various kinds. The purification of DNA from cancer tissues is an important step in diagnostic and therapeutic development, but current methods are not optimal. Many cervical cancer patients are also susceptible to high-risk human papillomavirus (HR-HPV) infection. Accurate viral diagnosis has so far relied on the extraction of adequate amounts of DNA from formalin-fixed, paraffin-embedded (FFPE) tissue samples. Since the sensitivity and specificity of commercially available purification kits are not optimal, we designed a DNA purification method based on AuNPs to purify sufficient amounts of HR-HPV DNA from cervical cancer tissue samples. AuNPs were coated with a series of oligonucleotide probes to hybridize to specific DNA sequences of HR-HPV genotypes. With this method, we recovered 733 out of 800 copies of type-specific HPV DNA with complete specificity, compared to 36 copies with a standard commercial kit (Qiagen FFPE).

Keywords

Biosensor; Cervical cancer; Gold nanoparticle; Human papillomavirus; Oligonucleotide probe; Paraffin embedding

1. Introduction

Gold nanoparticles (AuNPs) have advantageous characteristics of chemical addressability [1,2] and optical and electronic detectability that makes them useful in a wide variety of biosensor and therapeutic applications [3-7]. In particular, the facile attachment of oligonucleotide probe sequences (to give constructs designated here as Oligo-AuNPs) provides for high-affinity sensing or extraction of DNA in bacterial-based infections [8], parasitic diseases [9], viruses [10], and cancer biomarkers [11,12].

Among women, cervical cancer with a high incidence rate is significantly associated with high-risk human papillomavirus (HR-HPV) [13,14]. To investigate the role of HPV in cervical cancer, archived tissues from diagnostic pathology laboratories are a valuable resource [15]. Many such samples are preserved in the form of Formalin-Fixed, Paraffin-Embedded (FFPE) tissue, the standard method of preservation for many years [16]. While FFPE treated samples are very useful in immunohistochemistry (IHC) and hematoxylin and eosin (H&E) staining, they are difficult to analyze by molecular diagnostic methods such as microarray analysis and quantitative PCR because of significant damage to nucleic acids and formaldehyde-induced cross-linking in FFPE processing [17,18].

In order to extract nucleic acid from FFPE tissues, paraffin has to be removed or melted [19,20], giving rise to highly variable yields and quality of the extracted nucleic acid, which is highly dependent on sample age, fixation condition and size of tissue [21,22]. To overcome these problems, a number of methods and protocols have been developed to help extract nucleic acids from FFPE blocks, and to prepare it for downstream analyses [23-25]. Nearly all commercially available FFPE sample preparation and purification kits are designed to extract total RNA or DNA from the tissue, and a number of methods have been proposed to overcome problems experienced during these procedures [20]. We chose instead to develop a method to extract specific target sequences from such tissue, so as to increase the reliability, accuracy,

and sensitivity of PCR analysis. While HPV DNA can be specifically detected and amplified for diagnostic purposes using Oligo-AuNP probes [26,27], to our knowledge no protocols have been previously reported for the type-specific extraction of HPV DNA from FFPE tissue.

2. Results and discussion

2.1. Synthesis and characterization of Oligo-AuNPs

To begin the preparation of AuNPs bearing HR-HPV type-specific complementary oligonucleotide probes, spherical gold nanoparticles were synthesized by co-precipitation in the presence of sodium citrate, with size controlled by addition of sodium borohydride [28]. Synthesized AuNPs had UV-Vis spectra with a maximum peak at 510 nm (Figure 1). Morphology and size distribution of dispersed nanoparticles were evaluated by TEM analysis (Figure 3A). The obtained AuNPs were spherical and diameters of particles were measured in the range of 3 nm to 9 nm with a mean diameter of 5.1 nm (n=50). Dynamic light scattering was done to evaluate the hydrodynamic diameter of AuNPs. As shown in Figure 2, the diameter of nanoparticles was measured dominantly from 2.96 nm up to 15.19 nm. The AuNPs concentration (C_{AuNP}) was estimated from particle size (d = 5.1) and absorbance (A = 0.522) using theoretical relationship of eq (1), with C_1 = -4.75 and C_2 = 0.314 [29].

$$d = \left(\frac{A\left(5.89\times10^{-6}\right)}{C_{AuNP}\ exp(C_1)}\right)^{1/C_2} \quad (1)$$

C_{AuNP} was calculated as 2.13 µg/ml.

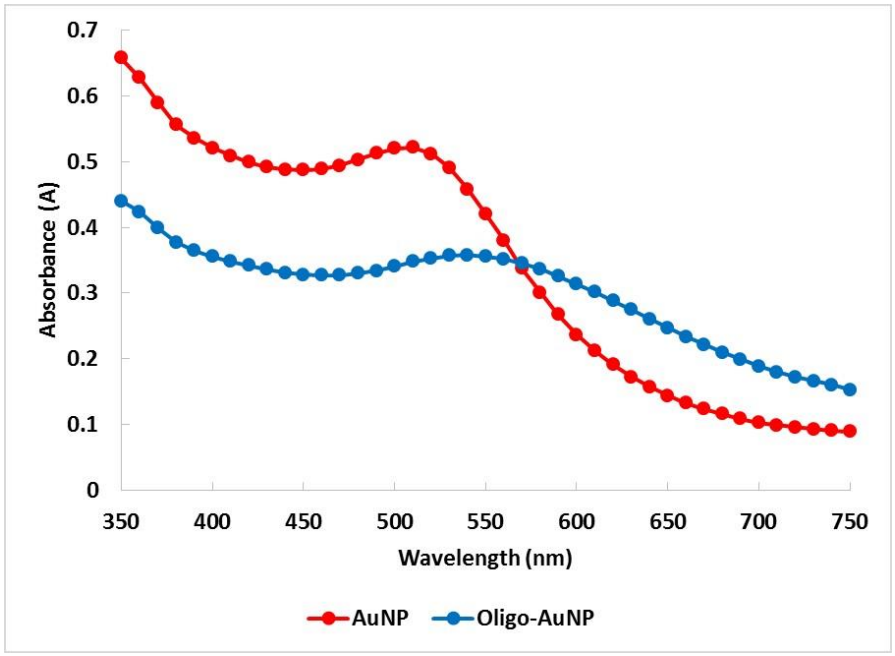
To discriminate HPV types 16, 18, 31, 33, 45, 52, and 58, five sets of oligonucleotide probes (35-39 nucleotides in length, designed to achieve a T_m for the binding of each probe to its complementary sequence of approximately 45°C) were created to target the variable L1 region of the HPV genome. One common probe was designed for the purification of HPV DNA types 18, 31, and 33, and the other four sets were designed for the purification of HPV DNA types

16, 45, 52, and 58. The sequences of all probes used are shown in Table S1 (see Supporting Information).

A thiol-terminated version of each oligonucleotide was obtained from commercial sources and was incubated with the AuNPs under standard conditions (PBS buffer, pH 7.4, 37°C) [30,31]. Unbound oligonucleotide probes were removed by washing twice with phosphate buffer (pH 7.0). Conjugation between AuNPs and the oligonucleotide probes resulted in clear supernatant with dark red oily precipitate. The red oily precipitate was dispersed in 0.05 M NaCl solution. Following interaction with the oligonucleotide, λ_{max} of AuNPs changed from 510 nm to 540 nm (Oligo-AuNP) (Figure 1). The efficiency of thiol-oligo binding to the particles was determined using the following equation:

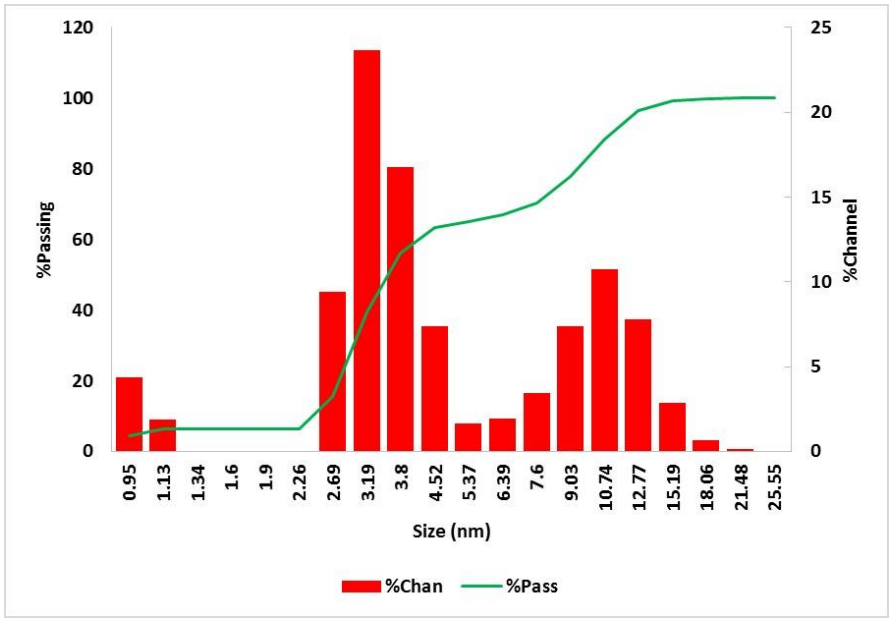
$$Efficiency = \frac{A0-A1}{A0} \times 100 \quad (2)$$

in which A0 is the absorbance of oligonucleotide probe ($OD_{260\ nm}$ = 0.746) and A1 is absorbance of the first obtained supernatant ($OD_{260\ nm}$ = 0.030). Uv-vis detection of unbound oligonucleotide in the supernatant showed nearly 95% of oligonucleotide probes to be attached to the gold nanoparticles (Figure 4). This maximum loading was obtained at an AuNP concentration of 1.06 µg/ml and thiol-modified oligonucleotide concentration of 20µM, performed in 20 µL volume. Minimal aggregation of oligo-AuNP particles was observed, with an average size increase to 12.6 nm diameter and a range of 6-22 nm (n=100, Figure 3B). The zeta potential of Oligo-AuNPs was found to be -137.7 mV compared to -67 mV before modification. It should be noted that other nanoparticle sizes have not been tested, and may not perform similarly [32]



121

122 **Figure 1,** UV-vis spectra of AuNP and AuNP probes.



123

124 **Figure 2,** Dynamic light scattering of AuNPs.

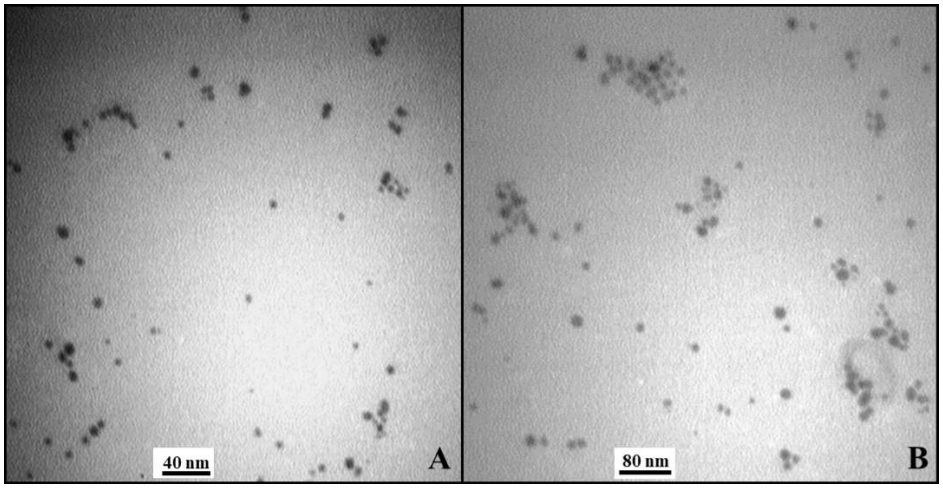


Figure 3, (A) TEM of AuNPs, (B) TEM of Oligo-AuNP.

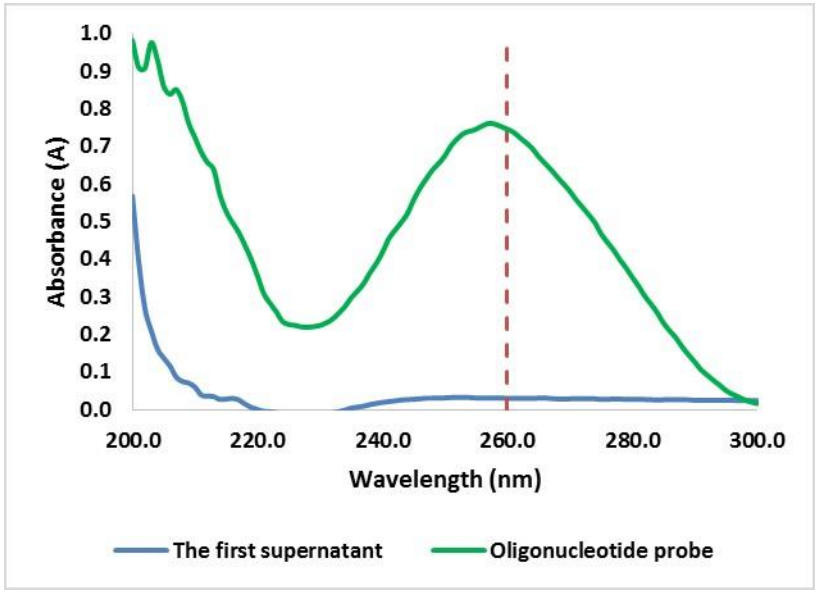


Figure 4, Optical density measurement for the calculation of loaded gold nanoparticles with oligonucleotide probes at wavelength of 260 nm.

2.2. Optimization of hybridization conditions

Hybridization for 30 minutes at a temperature of 45°C was found to be optimal for all probes after a series of tests at differing temperatures and annealing times (Figure 5), allowing the assay to be performed in a single tube containing a mixture of five sets of oligonucleotides.

Under these optimized conditions, a real-time PCR assay targeting a 145 bp of HPV L1 gene was able to detect 504 out of 800 copies/µL of purified HPV DNA with an efficiency of >90%.

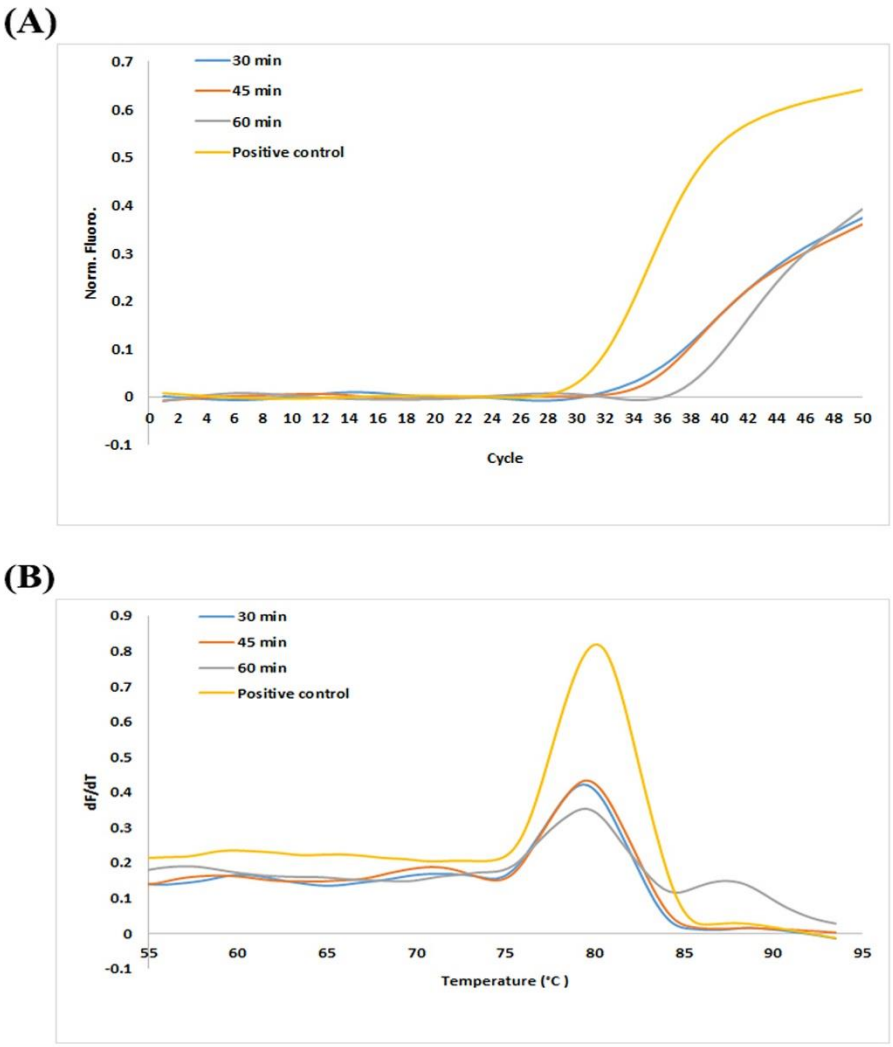


Figure 5, Optimization of hybridization time. **(A)** Effects of DNA purification incubation time on cycle threshold (Ct) values; (30 min: Ct=31.93), (45 min: Ct=33.16), and (60 min: Ct=36.95). Positive control = pHPV-16 DNA (**B)** Melting curve analysis of HPV-16 DNA at different times.

2.3. Performance verification of DNA purification method

DNA extraction, purification, and PCR efficiency have to be evaluated for each type of specimen, as these operations can be influenced by several experimental factors [33]. To monitor the DNA purification method efficiency, quantitative real-time PCR using

GP5+/GP6+ consensus primers was performed on a serial 10-fold dilution of HPV DNA genotypes 16, 18, 31, 33, 45, 52, and 58 ranging from 10^{10} copies to 1 copy per reaction.

PCR efficiency was determined from the slope of the standard curve for each genotype in a separate experiment. Quantitative PCR (qPCR) efficiency for genotypes 16, 18, 45, 52, and 58 were 89%, 90%, 91%, 92%, and 90% respectively which are considered acceptable for qPCR. The R2 values for all genotypes were 0.99 and the lower detection limit was 10 copies of HPV DNA/reaction.

The current gold standard for viral load is qPCR. However, various PCR-based quantitative methods such as quantitative PCR-enzyme immunoassay [34], TaqMan probes [35], FRET probes [36], AllGlo probes [37], and more recently digital droplet PCR have been used for the detection and quantification of HPV DNA extracted from tissue samples [38]. Since the accurate limit of detection and limits of quantification of a PCR method mainly depend on the DNA extraction step, it is difficult to compare the detection limit of our method with others. Generally, in terms of detection limit, droplet digital PCR assay is highly sensitive and has the theoretical detection limit of a single copy of target DNA.

2.4. Sensitivity evaluation of the DNA extraction methods

We compared our gold-nanoparticle based purification method with a standard Qiagen FFPE DNA kit, employing specimens with known HPV DNA concentrations. The AuNP probes gave rise to detection of 733 out of 800 copies (91.6%) of HPV-16 DNA, far in excess of the Qiagen kit sensitivity (35.7 of 800 copies, 4.5%, Figure 6). The threshold cycle value of DNA detection was thereby also lower for target DNA obtained from AuNPs than by Qiagen FFPE DNA kit extraction. The mean Ct value between AuNPs and the Qiagen kit in one case (blue) was as high as 10-fold differences, shown in Figure 6B. These differences are to be expected given

the lack of specificity in extraction by commercial tissue kit, and the targeted sequence extraction made possible by the use of specific probes on the AuNPs.

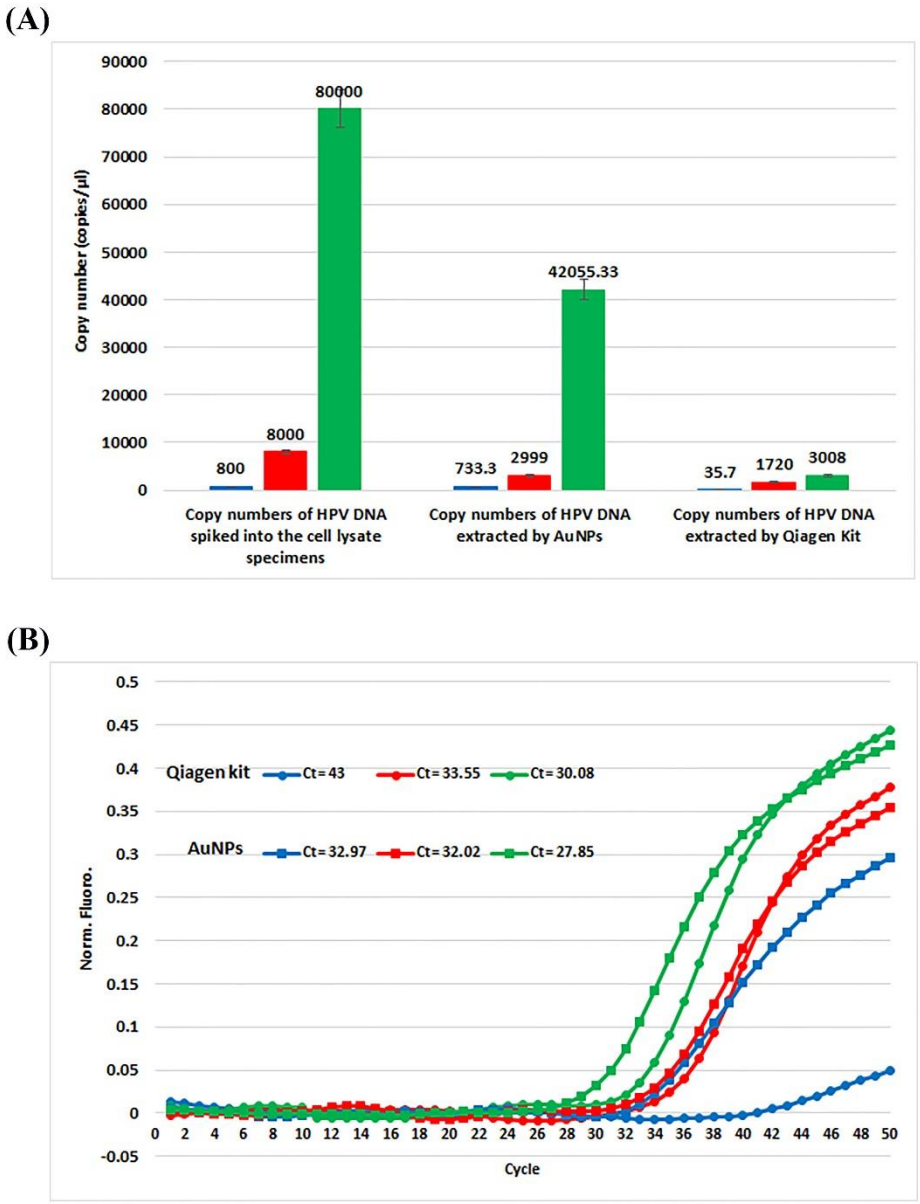


Figure 6, (A) Comparison of DNA purification efficiency after spiking HPV DNA (8×10^2 to 8×10^4) by AuNPs and Qiagen kit methods. **(B)** Threshold cycle (Ct) values for the amplification of the spiked HPV DNA recovered by two methods. (green: 8×10^4 copies/µL spiked HPV DNA, red: 8×10^3 copies/µL spiked HPV DNA, blue: 8×10^2 copies/µL HPV DNA).

178 The AuNP-probe method has the additional advantage of simpler workflow, requiring 5 steps
179 to obtain purified HPV-DNA as a template for PCR amplification as opposed to 21 steps in the
180 manufacturer's protocol for total DNA purification from FFPE tissue samples. In addition, the
181 traditional DNA extraction method requires xylene pretreatment for deparaffinization, which
182 is time-consuming and negatively affects the quality of extracted DNA. To overcome this
183 problem, we heated paraffin sections at 120°C prior to proteinase K treatment, resulting in
184 higher quality and quantity of DNA extract. Of course, these improvements are purchased at
185 the cost of specificity: our method cannot isolate DNA from papillomavirus, or any other
186 source, that does not match the probe sequences.
187
188 **2.5. Assessment of HR-HPV DNA purification in patient-derived samples**
189 To evaluate the performance of our method on FFPE cervical specimens, we compared FFPE
190 HPV genotyping results with the commercially available Ampliquality HPV-TYPE genotyping
191 assay which is based on single-step PCR and reverse line blot. Extracted DNA from 9 FFPE
192 cervical cancer tissue samples were tested for the presence of HR-HPV genotypes by two
193 methods. AuNPs-Probe was able to specifically isolate different HR-HPV genotypes (Table 1).
194 There was 100% concordance when the genotypes were tested by Ampliquality HPV-TYPE
195 genotyping assay, indicating excellent performance of AuNPs-Probe HPV DNA genotype
196 isolation method for FFPE specimens.
197 In addition, 9 HPV negative FFPE samples from benign ovarian tumor, and 4 randomly
198 selected FFPE tissue samples positive for low-risk HPV genotypes 6, 53, 87, and 90 previously
199 tested with Ampliquality assay as a reference method were examined. Real-time PCR assay
200 yielded negative results, indicating 100% specificity of the purification method. No false
201 positive was found in negative controls by newly designed AuNPs method. Samples showed
202 no cross-reactivity with the designed specific probes as well.

Table 1, Specificity of AuNPs-Probe purification method using FFPE cervical cancer tissue samples

No.	Genotype	Threshold cycle	DNA copies/reaction
1	HPV -16	33.27	2.14×10^2
2	HPV- 16	26.67	1.42×10^4
3	HPV-16	34.93	7.49×10
4	HPV-16	35.23	6.20×10
5	HPV-16	33.69	1.65×10^2
6	HPV-18	23.22	1.27×10^5
7	HPV-33	36.63	2.55×10
8	HPV-45	32.76	2.97×10^2
9	HPV-52	31.19	8.06×10^2

One drawback in using AuNPs in the PCR assay is its negative effect on DNA polymerase activity, probably due to its bondage with the enzyme [39,40]. To eliminate this problem, bovine serum albumin (BSA) in the concentration of 700 μg/ml and one unit of *Taq* DNA polymerase were added to the PCR master mix. Furthermore, the elution step was included in the procedure to separate the AuNP-Probe complex from the target DNA sequence to improve PCR efficiency [41].

3. CONCLUSION

A high yield DNA isolation method based on gold nanoparticle and probe hybridization assay was introduced to improve purification and detection of HR-HPV DNA in FFPE tissue of cervical cancer. The method was formed based on AuNPs coated with common or specific oligonucleotide probes complementary to HR-HPV L1 gene. The main strength of this method is the increased amount of purified DNA in comparison with the widely used QIAamp DNA FFPE Tissue Kit (91.6% vs. 4.5%). In addition, the method reduced number of steps in the procedure with a positive effect on the DNA yield. The developed method can be designed to specifically purify various DNA from different clinical samples. However, more samples have to be tested to evaluate its application in practice.

Abbreviations

HR-HPV: high-risk human papillomavirus; FFPE: formalin-fixed, paraffin-embedded; AuNPs: gold nanoparticles; IHC: immunohistochemistry; H&E: hematoxylin and eosin; C_{AuNP}: AuNPs concentration; $OD_{260\ nm}$: optical density at 260 nm; Ct: cycle threshold; qPCR: Quantitative PCR; BSA: bovine serum albumin; ATCC: American Type Culture Collection; HD: hydrodynamic diameter; DLS: dynamic light scattering; PBS: phosphate buffer saline; SSC: saline-sodium citrate; SDS: sodium dodecyl sulfate; RCC: Research Consultation Center.

Declarations

Ethics approval and consent to participate

The study was approved by the Research Ethics Committee of Shiraz University of Medical Sciences.

Consent for publication

Since this is a non-interventional, retrospective, subject-anonymized study, written patient consent was not required by the Research Ethics Committee.

Availability of data and materials

The datasets used and/or analyzed in the present study are available from the corresponding authors.

Competing Interests

The authors declare no competing financial interest.

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Author Contributions

All authors discussed the results and contributed to the final manuscript.

Abbas Behzad-Behbahani and Fatemeh Farjadian presented the original idea and designed the study. Noorossadat Seyyedi contributed to sample preparation and carried out the experiment. Noorossadat Seyyedi, Abbas Behzad-Behbahani, and Fatemeh Farjadian contributed to final version of the manuscript. Ali Farhadi and Gholamreza Rafiei Dehbidi supervised the experimental performance. Noorossadat Seyyedi and Negin Nikouyan took the lead in writing the manuscript. Reza Ranjbaran analyzed the data. Farahnaz Zare and Mohammad Ali Okhovat carried out the experiment in the pathology section.

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