

Research Paper

A Versatile Nanowire Platform for Highly Efficient Isolation and Direct PCR-free Colorimetric Detection of Human Papillomavirus DNA from Unprocessed Urine

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Abstract

Purpose: As human papillomavirus (HPV) is primarily responsible for the development of cervical cancer, significant efforts have been devoted to develop novel strategies for detecting and identifying HPV DNA in urine. The analysis of target DNA sequences in urine offers a potential alternative to conventional methods as a non-invasive clinical screening and diagnostic assessment tool for the detection of HPV. However, the lack of efficient approaches to isolate and directly detect HPV DNA in urine has restricted its potential clinical use. In this study, we demonstrated a novel approach of using polyethylenimine-conjugated magnetic polypyrrole nanowires (PEI-mPpy NWs) for the extraction, identification, and PCR-free colorimetric detection of high-risk strains of HPV DNA sequences, particularly HPV-16 and HPV-18, in urine specimens of cervical cancer patients. **Materials and Methods:** We fabricated and characterized polyethylenimine-conjugated magnetic nanowires (PEI/mPpy NWs). PEI/mPpy NWs-based HPV DNA isolation and detection strategy appears to be a cost-effective and practical technology with greater sensitivity and accuracy than other urine-based methods. **Results:** The analytical and clinical performance of PEI-mPpy NWs was evaluated and compared with those of cervical swabs, demonstrating a superior type-specific concordance rate of 100% between urine and cervical swabs, even when using a small volume of urine (300 μ L). **Conclusion:** We envision that PEI-mPpy NWs provide substantive evidence for clinical diagnosis and management of HPV-associated disease with their excellent performance in the recovery and detection of HPV DNA from minimal amounts of urine samples.

Key words: urine, cell-free DNA, HPV, cervical cancer, magnetic nanowires, colorimetric detection.

Introduction

Cervical cancer is the third most frequently diagnosed cancer and the fourth highest cause of cancer mortality among women globally [1, 2]. Cervical cancer exhibits an increased incidence in developing countries as a result of the absence of efficient and reliable screening services [3]. It is well

established that persistent infection and progression of high-risk oncogenic types of human papillomavirus (HPV) initiates an abnormal dysplasia of the squamous cells on the surface of the cervix, which increases the likelihood of developing cervical cancer [4, 5]. According to the International Agency

for Research on Cancer (IARC), ~25 strains of HPV are classified as high-risk oncogenic forms that are responsible for the incidence of cervical cell abnormalities or HPV-associated cancers. In such high-risk groups, HPV-16 and HPV-18 are the most common types and are found in ~70% of all cervical cancers [6]. Recently, the detection of urine-derived HPV DNA has opened up new clinical opportunities in developing applications for rapid detection and identification of HPV genotypes, offering great advantages over conventional methods such as the Pap smear test and liquid-based cytology [7-11]. In general, the standard screening method for cervical cancer involves invasive vaginal cytologic examinations that cause acute discomfort or pain to the patient. Moreover, such methods require specialized equipment and trained personnel, which is extremely difficult to establish and manage in resource-limited settings. However, urine as a potential alternative HPV source, allows for a simple, convenient, and efficient approach in collection and storage of the sample, as well as in the diagnosis of cervical cancer, which could contribute to maximizing the number of women receiving cervical screening tests. Recent studies provide the correlation results between urine and cervical samples by real-time PCR amplification, where the sensitivity and specificity of urine HPV testing were 68.6 % and 93.2 %, respectively, in comparison to those obtained in cervical samples [12].

Since the discovery of elevated levels of cell-free DNA (cfDNA) in the bodily fluids of cancer patients (i.e., whole blood, plasma, sputum, and urine), there has been rapid progress in technological development of non-invasive diagnostic and prognostic applications that enable real-time monitoring of cancer progression and response to therapies [13-16]. Efficient recovery and analysis of circulating DNA can provide valuable clinical insights to evaluate carcinogenic HPV detection and genotyping in urine samples. In this study, we introduce a novel strategy of urine-based HPV screening using polyethylenimine-conjugated magnetic polypyrrole nanowires (PEI-mPpy NWs), which play a crucial role in both isolating and detecting cfDNA in urine (Figure 1). Indeed, it is necessary to establish a standard protocol for optimal extraction of cfDNA in urine, because it affords great opportunity to develop reliable and comprehensive urine-based HPV testing. By employing this approach, we developed a strategy for ultrasensitive PCR-free colorimetric analysis of urinary HPVs with genetic variations without the need for any specialized equipment, which is important for rapid, non-invasive, and inexpensive point-of-care (POC) testing in resource-poor areas.

Results and Discussion

Magnetic nanowires can serve as a versatile platform for extracting large quantities of cfDNA from urine as well as detecting target HPV genes in colorimetric recognition with great accuracy and sensitivity. The polycation polyethylenimine (PEI) offers the advantage of having very high affinity for negatively-charged DNA by readily forming nanowire-DNA complexes through electrostatic interactions [17-21]. We applied this cationic nature of PEI for the rapid and efficient isolation of cfDNA from urine with high yield and purity, and for direct PCR-free detection of target HPVs via the addition of multiple horseradish peroxidase (HRP)- and streptavidin-labelled polypyrrole (Ppy) nanoparticles (HRP/st-tagged NPs) to greatly magnify colorimetric signals (Figure 1A; PEI-mPpy NWs). Initially, Ppy nanowires were electrochemically deposited within the pores of the anodic aluminum oxide (AAO) template in which biotin and Fe₃O₄ magnetic nanoparticles (MNPs; 10 nm in diameter) were employed as co-dopants. Using an AAO membrane as a sacrificing layer offers particular advantages in that it allows not only precise manipulation of the diameter and length of the nanowires, but also ensures sufficient accumulation of a high density of biotin and magnetic nanoparticles in the channels of the template. Field emission scanning and transmission electron microscopy (SEM and TEM, respectively) images provided detailed characterization of PEI-mPpy NWs showing an average diameter of 200 nm and a mean length of 18 μm (Figure 1B, Left). High-magnification TEM imaging confirmed the presence of ~10 nm Fe₃O₄ MNPs in the nanowires with random orientation and densely packed distribution (Figure 1B, Middle). In addition, the morphology of HRP/st-tagged NPs was observed by SEM (Figure 1B, Right). On the other hand, PEI-conjugated magnetic Ppy nanowires (PEI-mPpy NWs) displayed a relatively high saturation magnetization of 87 emu/g, whereas no magnetization saturation and no hysteresis features were observed in PEI-conjugated Ppy nanowires (PEI-Ppy NWs) (Supporting Figure S1).

It is likely that the incorporation of high amounts of magnetic nanoparticles in the nanowires is strongly correlated with the enhanced magnetization behavior of the nanowires. Recently, urine-based HPV screening approaches have been widely investigated as a potential alternative to conventional cytology tests to detect cervical cancer [22-24]. Indeed, as urine represents an easily accessible specimen, it readily allows simple and non-invasive self-collection of large amounts with repeated sampling. We therefore anticipate that, with advances in isolating and

detecting urine-derived cfDNA implicated in the development of cervical carcinogenesis, urine testing could be a more reliable and effective tool for identifying and monitoring women who are at risk for cervical cancer. However, HPV detection by urine is particularly difficult to implement because HPV DNA is found at extremely low levels in urine and inappropriate extraction protocols are often employed. The lack of adequate strategies in isolating cfDNA from urine renders the urinary HPV detection rate relatively low and sometimes unsuccessful compared to that from conventional cervical swab samples [25, 26]. The development of DNA extraction protocols yielding sufficiently high quality and quantity of DNA is the most crucial step in the process of the molecular analyses that subsequently ensures high-performance PCR-free colorimetric detection of HPVs with diverse genetic variations. Accordingly, the first step in enhancing the accuracy and reliability of urine-based HPV DNA testing is to maximize the isolation efficiency of cfDNA from urine. Taking this consideration into account, the thin and extended features of the nanowires are likely to improve recovery efficiency by greatly increasing the interaction frequency and duration of HPV DNA present in urine. We expect that the recovery of elevated levels of cfDNA from urine directly contributes to a high HPV type-specific concordance between urine and cervical swab samples. Therefore, we validated the capability of PEI-mPpy NWs that were conjugated with different molecular weights of branched PEI (800 Da and 25 kDa) through *ex vivo* spiking with a known concentration of genomic DNA (gDNA) from HPV-positive cell lines (HeLa: HPV-18-positive, and SiHa: HPV-16-positive cells) into the HPV-negative urine pool (Supporting Figure S2A-B).

Higher DNA recovery was achieved with >90% isolation efficiency by using PEI-mPpy NWs labeled with 25 kDa PEI across a range of concentrations of input gDNA. Meanwhile, the efficacy of PEI-mPpy NWs conjugated with low molecular weight PEI (800 Da) was markedly decreased with increasing concentrations of gDNA. We found that PEIs with a higher molecular weight (25 kDa) exhibited better binding and condensation ability with DNA molecules than low-molecular-weight PEIs (800 Da), likely due to the fact that complex formation by electrostatic interactions is strongly dependent on the molecular weight of PEIs, which is consistent with previous studies [19, 21]. Using quantitative real time polymerase chain reaction (qPCR), we subsequently analyzed the limit of detection (LOD) of various concentrations of genomic DNA from HPV-positive cell lines (HeLa: HPV-18-positive, and SiHa:

HPV-16-positive cells) that were *ex vivo* spiked into the HPV-negative urine pool. Generally, the LOD was defined as the lowest concentration of HPV DNA detected with positive test results of at least 95% based on threshold cycle (Ct) values, which were compared with those from the Roche Cobas 4800 HPV test. The analytical sensitivity (LOD) of PEI-mPpy NWs in the cfDNA isolated from urine was 0.5 pg/ μ L for HPV-16-positive SiHa cells and 1.2 pg/ μ L for HPV-18-positive HeLa cells, whereas the LOD for the Roche Cobas HPV test was 5.2 pg/ μ L for HPV-16-positive SiHa cells and 1.6 pg/ μ L for HPV-18-positive HeLa cells, indicating that the nanowires showed substantially improved analytical performance compared to the values reported in previous studies (Supporting Figure S2C) [2]. The feasibility of PEI-mPpy NWs in HPV DNA isolation was further examined using urine samples of ten representative patients with cervical cancer. Initially, we attempted to measure and compare the concentration of cfDNA isolated by using PEI-mPpy NWs (black bars) and a Qiagen DNA extraction kit (red bars) (Figure 2).

Interestingly, the total amount of DNA obtained from urine using PEI/mPpy NWs was approximately 4-fold higher than that obtained using the Qiagen kit. Most importantly, an elevated level of eluted cfDNA appears to be closely relevant to the accuracy of HPV DNA detection and genotyping, as shown in Table 1. For this study, HPV genotyping profiles of cervical cancer patients were established from direct swab samples of cervical tissue and confirmed by the Roche Cobas 4800 HPV Test with high-risk HPV types (HPV-16, -18, and -others). Paired urine samples were applied to PEI-mPpy NWs and Qiagen reagents to efficiently isolate HPV DNA and elucidate their genotype profiles by qPCR. Of the 15 HPV-positive samples, HPV DNA was identified in all 15 (100%) when isolated by using PEI-mPpy NWs, whereas HPV DNA was detected in 5 urine samples (33.3%) when extracted with the Qiagen kit. Indeed, HPV DNA recovered by the PEI-mPpy NWs exhibited obviously lower cycle threshold (Ct) values compared to that from the Qiagen kit, which strongly suggests higher performance of the nanowires in DNA recovery yield and integrity. Significant enhancement in the overall yield of target HPV in urine samples could be attributed to the elongated shape of the nanowires, which allows a large number of interactions with HPV DNA while retaining bioactivity. Indeed, it is likely that the pencil-like shape of the nanowires not only gives them a large surface-to-volume ratio, but also enables them to move freely through the complex urinary constituents, and thus these nanowires are capable of

binding preferentially to cfDNA due to increased duration and frequency of exposure. Next, we attempted to isolate genomic DNA (HPV-positive SiHa cells (HPV-16) and HeLa cells (HPV-18); 250 ng/mL) *ex vivo* spiked into the HPV-negative urine pool using the PEI-mPpy NWs and utilized them (i.e., nanowire-DNA complexes) as a template for the PCR-free detection of HPVs via a novel colorimetric signal amplification strategy. Without the PCR amplification step, detection occurs following hybridization of biotin-labelled capture probe (CP) and detector probe (DP) with its corresponding target HPV DNA in the nanowire-DNA complexes, as shown in Figure 3A.

Indeed, both probes sequentially hybridize to their complementary HPV sequences on the nanowire-DNA template. After hybridization of the probe to its target sequence, multiple horseradish peroxidase (HRP)- and streptavidin-labeled polypyrrole nanoparticles (HRP/st-tagged NPs) were added for the specific recognition, with capture and detector probes linked with biotin in a solution containing 1 mM colorimetric TMB substrate and 0.5 mM H₂O₂. Ultimately, we were able to visualize the colorimetric signals that were derived from the specific interaction of the probes with their respective target HPV sequences [27, 28]. The addition of HRP/st-tagged NPs obviously facilitates H₂O₂-mediated catalytic oxidation of TMB, which triggers substantial color change in the presence of target DNA [26]. Under optimal experimental conditions, we evaluated the dynamic range of colorimetric UV signals using the calibration plots that were strongly correlated with the concentration of the target DNA, exhibiting a linear relationship between the absorbance value of the oxidized TMB at 650 nm and the concentration of the target DNA, with detection limits of 0.052 pg/μL (HPV-16-positive SiHa cells) and 0.12 pg/μL (HPV-18-positive HeLa) based on a signal-to-noise ratio of 3. The magnetic nanowires are optimally suitable for the isolation of cfDNA from urine at high efficiency, and thus can be a key component in improving the workflow of routine urine-based HPV DNA analysis. The clinical utility of the PCR-free colorimetric assay was further evaluated by testing urine samples that were collected from cervical cancer patients and healthy controls (Figure 4).

Firstly, cfDNA was extracted from urine samples with a volume <300 μL from HPV18-positive cancer patients, HPV16-positive cancer patients, and HPV-negative healthy controls by using PEI-mPpy NWs, where HPV genotyping had already been confirmed by standard cervical swab clinical examinations. PCR-free optimized procedures were

applied for *in situ* detection of HPVs in the nanowire-DNA complexes following these protocols: i) the denaturation of nanowire-DNA complexes at 95 °C for 1 min, ii) the introduction of complementary CP/DP to nanowire-DNA complexes for target-induced hybridization, and iii) the addition of HRP/st-tagged NPs to dramatically amplify the colorimetric signals. As shown in Figure 4A, two HPV18-positive urine samples displayed strong visible colorimetric response through the specific hybridization with HPV18 probes, whereas PBS, HPV16-positive, and HPV-negative urine samples showed no color change, confirming specific binding of their complementary probes with high sensitivity and specificity. Similarly, four HPV16-positive urine samples yielded a significant increase in colorimetric response upon exposure to HPV16 probes. However, all HPV16-negative samples including PBS, HPV18-positive, and HPV-negative urine samples did not respond to HPV16 probes. As expected, these results were in good agreement with absorbance values obtained by UV-Vis absorption spectroscopy (Figure 4B). Indeed, HPV18-positive urine samples were found to be highly specific and hybridized only in the presence of HPV18 probes, exhibiting strong absorption peaks at 650 nm even without PCR amplification. The quantitative colorimetric measurements showed that HPV16- and HPV18-positive urine samples yielded appreciably higher absorbance with their corresponding probes, but exhibited significantly low cross-reactivity with other HPV genotypes. On the other hand, HPV16-/18-negative urine samples and PBS resulted in negligible colorimetric signals in both probe types. We further evaluated the proposed nanowire-based strategy for ultrasensitive colorimetric determination of the HPV genotype distribution patterns using urine samples of cervical cancer patients and healthy controls, and ultimately compared the results with those from cervical swab samples. Initially, we compared the amount of the cfDNA extracted from a total of 28 urine samples (24 HPV-positive and 4 HPV-negative samples) of healthy controls and patients with cervical cancer by using PEI-mPpy NWs (Figure 5A).

We found that there were no apparent differences in the concentration of the eluted cfDNA between healthy individuals and HPV-positive cancer patients. We also investigated type-specific HPV detection in paired conventional swab sampling and urine testing (300 μL). Surprisingly, PEI-mPpy NWs greatly improved extraction and detection efficiency of urinary cfDNA with an excellent genotyping concordance rate (100%) between cervical swabs and urine-based colorimetric detection (Figure 5B, Table

2). Continually, we identified multiple serial HPV genotypes in the same nanowire-DNA complexes. The cfDNA extracted by PEI-mPpy NWs can serve as a versatile sensing platform for inducing specific recognition and binding of the probe to complementary DNA based on the colorimetric assay (Figure 6A). With the addition of a series of type-specific CP/DP and HRP/st-tagged NPs continuously in the same nanowire-DNA complexes that were generated from the urine of patients with both HPV16 and 18, we identified and discriminated multiple HPV targets with genetic diversity using one nanowire-DNA template. As shown in Figure 6B, we observed obvious color change and UV-Vis

absorbance at 650 nm of the nanowire-DNA complexes after adding the first target probe (i.e., HPV16 probes) and HRP/st-tagged NPs. However, as expected, no response was observed with the addition of EGFR19 probe to the same template. Immediately, a second target probe (i.e., HPV18 probes) and HRP/st-tagged NPs were added to the same nanowire-DNA complexes that confirmed the successful colorimetric response of specific HPV DNA segments with excellent sensitivity and specificity, even in repeated detection. Finally, the addition of the EGFR21 probe confirmed that no reaction occurred involving the nanowire-DNA template.

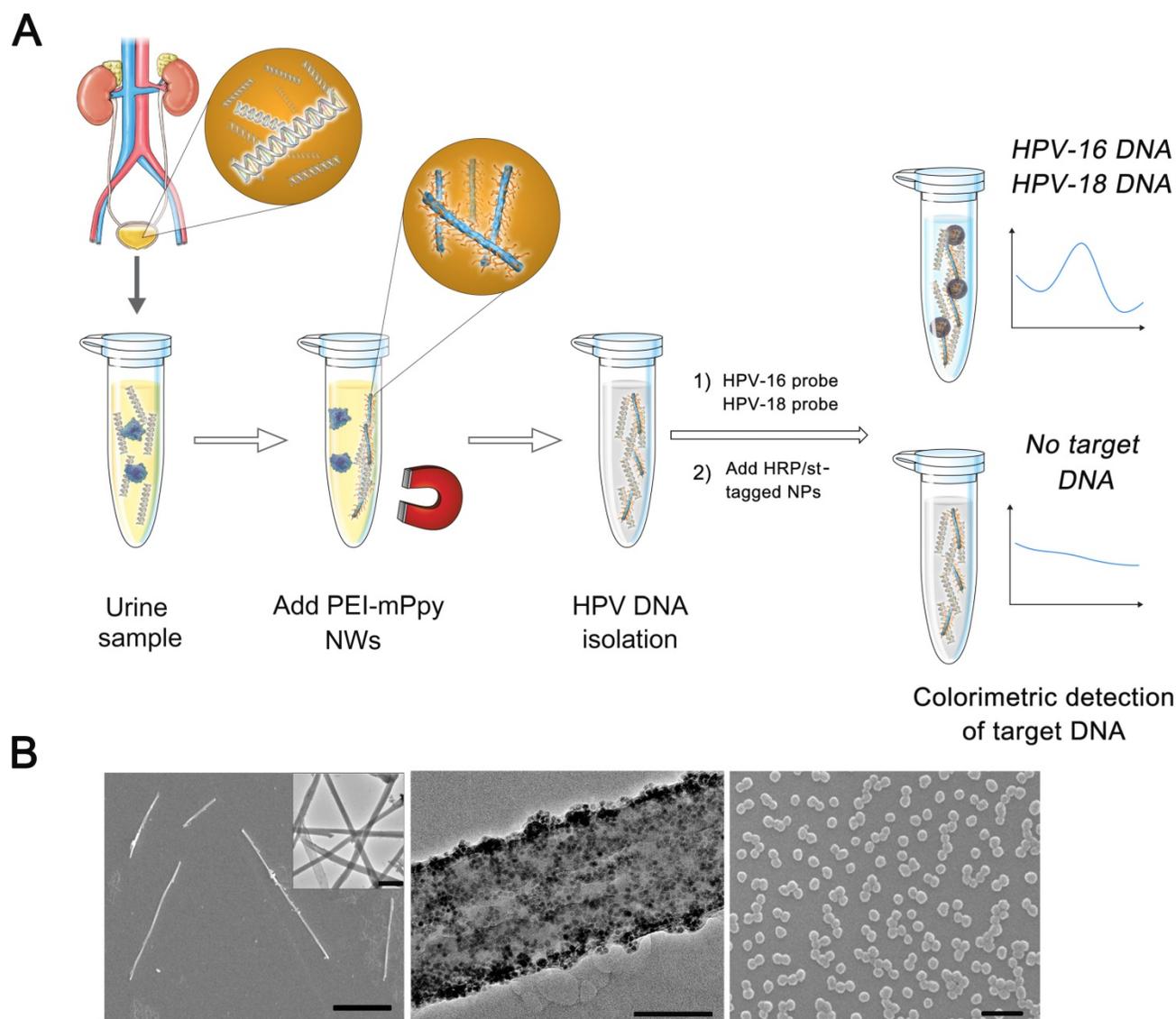


Figure 1. (a) Schematic diagram of highly efficient isolation of urinary cfDNA using polyethyleneimine-conjugated magnetic nanowires (PEI-mPpy NWs) and direct PCR-free colorimetric detection of target HPVs via sequential addition of complementary probes and multiple horseradish peroxidase (HRP)-streptavidin-labelled polypyrrole nanoparticles (HRP/st-tagged NPs) to dramatically amplify colorimetric signals. (b) SEM image of PEI-mPpy NWs (Left, Scale bar = 10 μm). Inset is a TEM image of the nanowires (scale bar = 1 μm). TEM image at high magnification showing the presence of a large quantity of magnetic nanoparticles (MNPs; 10 nm) doped within the nanowire (Middle, Scale bar = 50 nm). SEM image of HRP/streptavidin-conjugated nanoparticles (Right, scale bar = 200 μm)

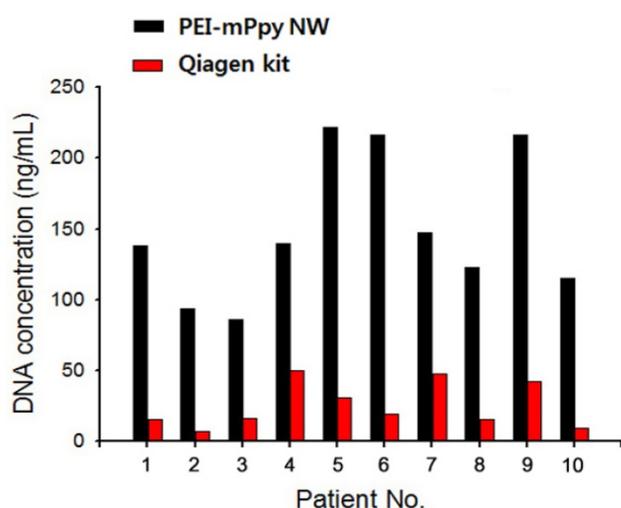


Figure 2. Validation of PEI-mPpy NWs in the extraction of cfDNA in urine samples of cervical cancer patients. Comparisons of the concentration of cfDNA isolated from urine samples of ten representative cervical cancer patients by using PEI-mPpy NWs and a Qiagen DNA extraction kit. PEI-mPpy NWs and a Qiagen kit were employed for the extraction of cfDNA from urine.

Table 1. The detection and genotyping of HPV DNA in cervical swabs and urine samples of cervical cancer patients.

Patient No.	Cervical swab HPV type	Urine HPV type		
		PEI-mPpy NW (Ct)	Qiagen kit (Ct)	Δ Ct
P1	HPV16	HPV16 (34.2)	N/A	-
P2	HPV16	HPV16 (37.2)	N/A	-
P3	12 other types	12 other types (29.6)	12 other types (33.9)	4.3
P4	HPV16	HPV16 (34.4)	N/A	-
P5	12 other types	12 other types (32.8)	12 other types (36.8)	4.0
P6	12 other types	12 other types (32.7)	12 other types (35.2)	2.5
P7	12 other types	12 other types (37.2)	N/A	-
P8	12 other types	12 other types (34.6)	12 other types (40.0)	5.4
P9	12 other types	12 other types (28.2)	12 other types (29.7)	1.5
P10	HPV16	HPV16 (33.9)	N/A	-
P11	12 other types	12 other types (30.7)	N/A	-
P12	12 other types	12 other types (31.0)	12 other types (40.0)	9.0
P13	HPV16	HPV16 (33.4)	HPV16 (38.3)	4.9
P14	HPV16	HPV16 (34.2)	N/A	-
P15	HPV18	HPV18 (31.6)	N/A	-

Conclusions

We have successfully evaluated the capabilities of PEI-mPpy NWs as a versatile platform for the isolation of HPV cfDNA from urine of cervical cancer patients, and for the identification and detection of multiple type-specific HPVs via direct PCR-free colorimetric signal amplification. The elongated features of the nanowires offer great advantages in enhancing the extraction yield of cfDNA by significantly increasing the interactions with DNA molecules in urine, thus promoting the ability for directly detecting the target HPV DNA from a small volume of urine (300 μ L) even without PCR amplification step. Indeed, colorimetric results can even be discriminated by the naked eye. Our PEI-mPpy NWs-based HPV DNA isolation and

detection strategy appears to be a cost-effective and practical technology with greater sensitivity and accuracy than other urine-based methods. These findings suggest that the use of PEI/mPpy NWs in urine-based HPV screening shows significant advancement in the isolation, identification, and analysis of multiple high-risk genotypes of HPV DNA, which provides a promising direction in non-invasive screening and detection of multiple genotypes in HPV-related cancers.

Experimental Section

Chemicals and reagents

Polyethyleneimine (PEI, branched, average MW ~25,000), iron oxide (II, III), magnetic nanoparticles solution (MNP, 10 nm average particle size, 5 mg/mL in H₂O), N-(3-dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride (EDC), N-hydroxy succinimide (NHS), pyrrole, biotin, and streptavidin were purchased from Sigma-Aldrich (St. Louis, MO, USA). An anodized aluminum oxide (AAO) membrane filter (200 nm pore diameter) was obtained from Whatman (Pittsburgh, PA, USA). For real-time PCR analysis, the Cobas 4800 HPV controls and amplification/detection kits were purchased from Roche Molecular Diagnosis (Pleasanton, CA, USA).

Preparation and characterization of PEI/mPpy NWs

Polyethyleneimine-conjugated magnetic nanowires (PEI/mPpy NWs) were fabricated as previously described [17]. Briefly, 30 μ L of magnetic nanoparticles (MNPs, 5 μ g/mL; ~10 nm in diameter) were deposited on top of the Au-coated Anodic Aluminum Oxide (AAO) membrane (~150 nm thick) and infiltration into the AAO nanopores was promoted by moderate aspiration at room temperature (RT). All electrochemical techniques were conducted using a potentiostat/galvanostat (SP-150, BioLogic, USA). Ppy was electrochemically polymerized in the MNP-loaded AAO template nanochannels in a solution of 0.01 M poly(sodium 4-styrenesulfate) containing 0.1 M pyrrole and 1 mM biotin by applying chronoamperometry at 1.0 V (vs. Ag/AgCl) for 7 min. The resulting AAO templates were rinsed several times with distilled water, immersed in 2 M NaOH for 3 h, and then placed in a sonication bath (Bioruptor® UCD-200, Diagenode Inc, USA) to obtain individual, free-standing Ppy NWs. Then, 30 mM N-(3-dimethylaminopropyl)-N-ethylcarbodiimide hydrochloride (EDC) and 6 mM N-hydroxysuccinimide (NHS) were added to the resulting Ppy NWs to activate carboxylic acid groups. Subsequently, the resulting nanowires were

incubated with streptavidin (10 $\mu\text{g}/\text{mL}$) for 45 min and washed with distilled water. Then, biotinylated PEI (800 or 25000 Da) was added to the streptavidin-labeled nanowires at RT for 1 h. The morphologies of the PEI/mPpy NWs were analyzed

by SEM (JSM-7800F, JEOL, USA) and TEM (G2 F30ST, Tecnai, USA). Magnetic field measurements were investigated by a SQUID-VSM magnetometer (MPMS 3, Quantum Design, USA).

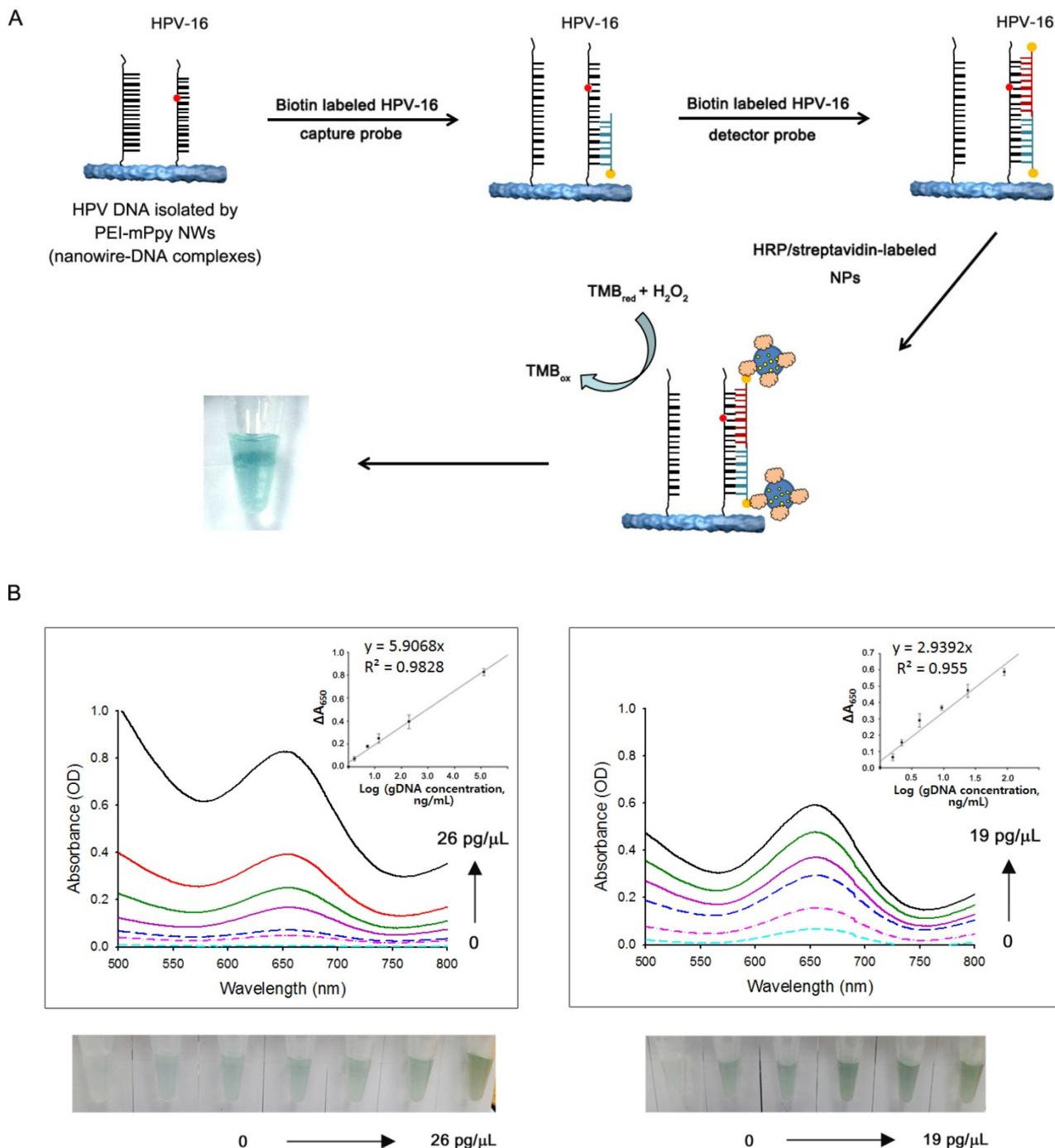


Figure 3. (A) Magnetic nanowire-based colorimetric assay for HPV DNA detection and genotyping in urine samples. Colorimetric detection was performed on the nanowire-DNA complexes that contained cfDNA isolated from the urine samples using PEI-mPpy NWs. The biotin-labeled capture and detector probes were specifically designed to recognize the corresponding target HPV DNAs attached to the nanowire, even without a PCR amplification step. After hybridization of the target with type-specific probes, multiple horseradish peroxidase (HRP)- and streptavidin-labeled polypyrrole nanoparticles (HRP/st-tagged NPs) were added to produce amplified colorimetric signals that are even visible to the naked eye. (B) UV-Vis absorption spectra of nanowire-DNA complexes that are hybridized specifically with their complementary capture/detector probes followed by the addition of HRP/st-tagged NPs. Known concentrations of genomic DNA from HPV-positive SiHa cells (Left; HPV-16; 0, 0.16, 0.52, 1.3, 2.6, 5.2, 26 $\mu\text{g}/\mu\text{L}$) and that from HPV-positive HeLa cells (Right; HPV-18; 0, 0.12, 0.4, 1.8, 3.9, 19 $\mu\text{g}/\mu\text{L}$) were spiked into HPV-negative urine pool *ex vivo*, for HPV DNA isolation and colorimetric detection. The insets show plots of the corresponding absorbance at 650 nm versus various concentrations of genomic DNA from HPV-positive SiHa cells (Left; HPV-16) and HeLa cells (Right; HPV-18) that were extracted by PEI-mPpy NWs. The error bars represent the standard deviations from five independent measurements.

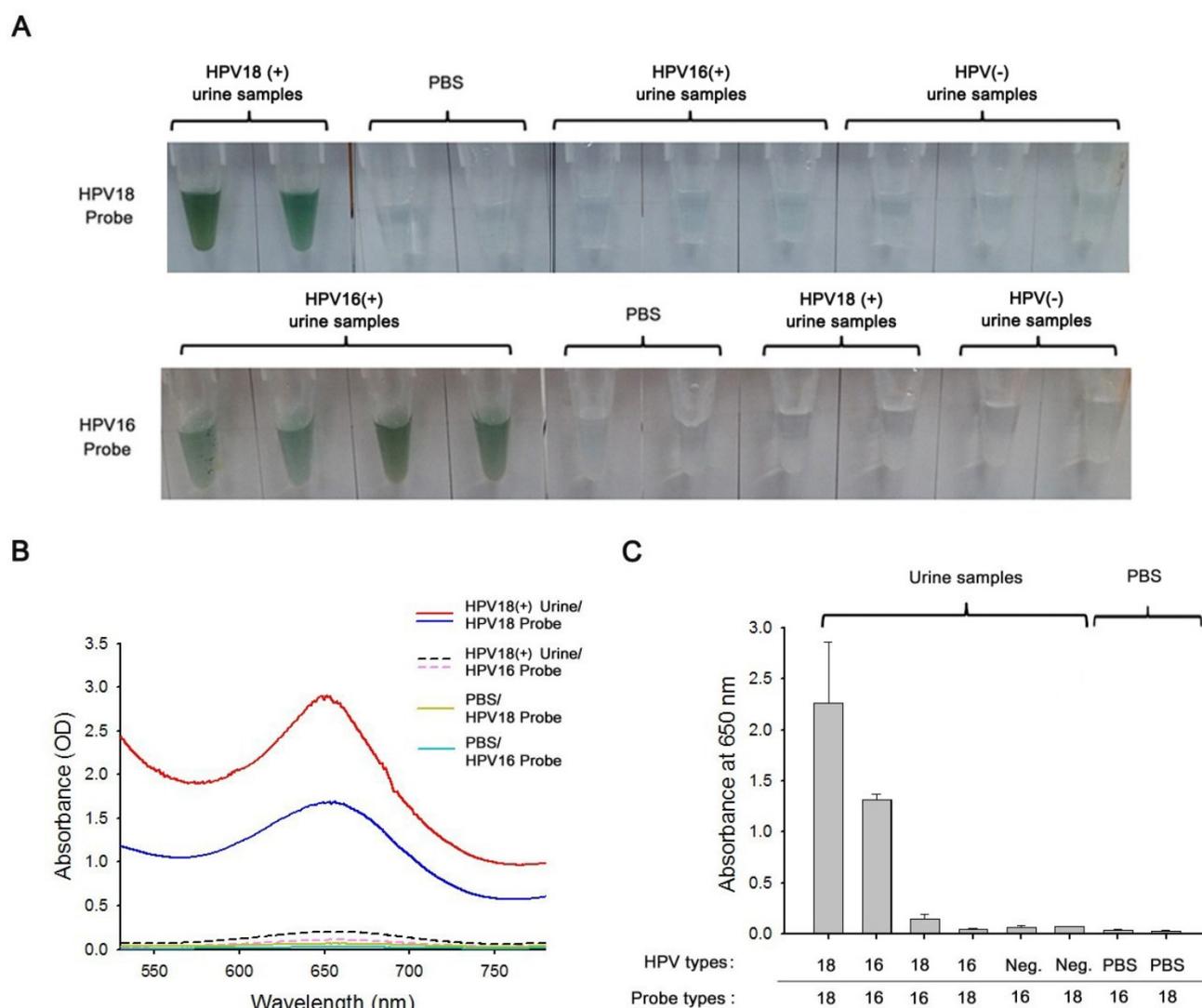


Figure 4. (a) Assessment of clinical performance of the proposed PCR-free colorimetric assay by evaluating urine samples of HPV-positive cervical cancer patients (HPV16(+)) and HPV18(+)), HPV-negative healthy controls (HPV(-)), and PBS. The photographs display the color change of nanowire-DNA complexes after hybridization with biotin-labelled capture and detector probes with the corresponding target HPV DNA isolated using the nanowire, followed by the addition of HRP/st-tagged NPs. (b) The UV-Vis spectra of nanowire-DNA complexes that contain cfDNA isolated from HPV18-positive urine by PEI-mPpy NWs. With the addition of HPV16/HPV18 probes and HRP/st-tagged NPs, type-specific HPVs can be specifically detected. (c) Average absorbance values of circulating cfDNA isolated from urine samples of HPV-positive cervical cancer patients (HPV16(+)) and HPV18(+)), HPV-negative healthy controls (HPV(-)), and PBS after the reaction with different probe types specific for HPV16 or HPV18. A total of 24 HPV-positive and HPV-negative urine samples were collected and tested. The error bars represent the standard deviations from five independent measurements.

Urine Samples

Urine samples were obtained from thirty HPV-positive patients and five healthy volunteers at the National Cancer Center Hospital (Goyang, Korea) according to procedures approved by the NCC Institutional Review Board (NCC2016-0282). The collected urine samples were immediately aliquoted and stored at -20°C until use.

Evaluation of genomic DNA capture and release efficiency by using PEI-mPpy NWs

Defined quantities of genomic DNA from HPV-positive cell lines (HeLa: HPV-18-positive and SiHa: HPV-16-positive cells) were *ex vivo* spiked into a

HPV-negative urine pool at concentrations ranging from 0.01 to 1,000 ng/mL. Subsequently, DNA capture was carried out by adding PEI/mPpy NWs (0.5 mg/mL) to the urine samples (300 μL) containing the genomic DNA with gentle shaking (120 \times g) for various time durations (10, 30, 60, and 90 min) at RT. Then, the resulting solutions were placed onto a magnetic rack for 30 min to separate unbound or non-specifically bound constituents and capture DNA-bound magnetic nanowires. The captured DNA was then eluted in 10 mM Tris-HCl buffer (pH 10.0) by maximum speed shaking (750 \times g) for various times (10, 30, 60, and 90 min) at RT. The captured and released DNA was quantified by PicoGreen assay

mixed for 30 min to capture cfDNA. After being precipitated in PBS, the resulting cfDNA-magnetic nanowires were denatured at 95°C for 1 min and hybridized with 1 pM biotin-terminated HPV-16-CP and HPV-16-DP for 1 h at 37°C. Next, the HRP/st-tagged NPs were added to the samples and incubated for 30 min at 37°C. To the resulting solution, 25 μL of 10 mM 3,3',5,5'-Tetramethylbenzidine (TMB), 25 μL of 0.1 M H₂O₂, and 200 μL of 0.2 M sodium acetate trihydrate buffer (pH 5.0) were added to

observe the colorimetric signals. After that, the DNA captured by the nanowires were consecutively incubated in a solution containing biotin-terminated EGFR 19-, HPV-18-, or EGFR 21- probes, followed by the addition of the HRP/st-tagged NPs and 25 μL of 10 mM 3,3',5,5'-Tetramethylbenzidine (TMB) in 200 μL of 0.2 M sodium acetate trihydrate buffer (pH 5.0) containing 25 μL of 0.1 M H₂O₂, and ultimately the colorimetric signals were detected using the same procedure as described previously.

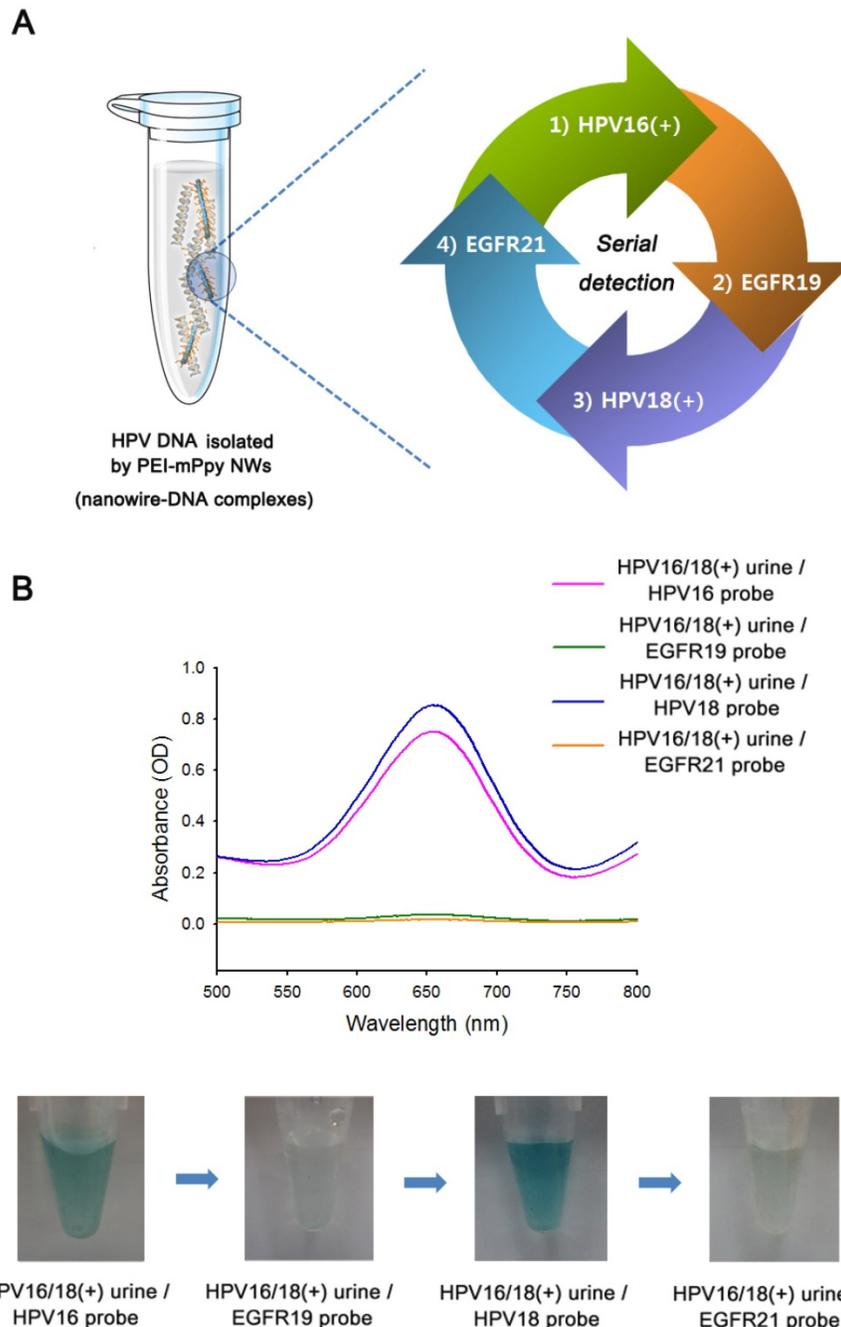


Figure 6. (a) A novel approach of PEI-mPpy NWs in the extraction, identification, and PCR-free sequential detection of multiple HPV genotypes from urine specimens of cervical cancer patients. (b) The UV-Vis colorimetric results of cfDNA isolated by PEI-mPpy NWs from urine of cervical cancer patients who were found to be positive for both HPV16 and HPV18, demonstrating multiple uses of the same nanowire-DNA complexes for the detection of HPV with different genetic variations. However, no response was observed for non-HPV probes (EGFR19 and EGFR21). The photographs indicate the color change as a result of type-specific hybridization between target HPVs and their complementary probes.

Table 3. HPV capture and detector probe sequences

Probe label	Sequences
HPV 16 - CP	5' biotin - GAG GAG GAG GAT GAA ATA GAT GGT - 3'
HPV 16 - DP	5' - TTG GAA GAC CTG TTA ATG GGC - biotin 3'
HPV 18 - CP	5' biotin - CAC ATT GTG GCA CAA TCT TTT A - 3'
HPV 18 - DP	5' - GCC ATA TCG CTT TCA TCT GT - biotin 3'
EGFR 19 - CP	5' biotin - GGAATTAAGAGAAGCAACATCTCC -3'
EGFR 19 - DP	5' - AACCTCAGGCCACCTTTT - biotin 3'
EGFR 21 - CP	5' biotin - CCAGGAACGTACTGGTGAAAA - 3'
EGFR 21 - DP	5' - GGAAGAGAAAGAATACCATGCA - biotin 3'

Abbreviations

HPV: human papillomavirus; EGFR: epidermal growth factor receptor; PEI-mPpy NWs: polyethylenimine-conjugated magnetic polypyrrole nanowires; cfDNA: cell-free DNA; POC: point-of-care; PCR: polymerase Chain Reaction; HRP: horseradish peroxidase; AAO: Anodic Aluminum Oxide; Ppy: polypyrrole; CP: capture probe; DP: detector probe; TMB: 3,3',5,5'-Tetramethylbenzidine.

Supplementary Material

Supplementary figures.

<http://www.thno.org/v08p0399s1.pdf>

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Author contributions

S.J., H.L., and Y.C. designed the study and analyzed the data. S.J. and H.L. fabricated the platforms and performed the DNA capture and release experiments. K.B. and K.Y. collected and analyzed the digital PCR data. K.Y. and E.L. prepared the clinical samples. S.J., H.L., K.Y., and Y.C. co-wrote the manuscript. All authors discussed the results and the comments on the manuscript.

Competing Interests

The authors have declared that no competing interest exists.

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