Target-mediated Rolling Circle Transcription Coupling with CRISPR/Cas12a-Cas13a for Simultaneous Detection of HPV16 and HPV18

Shiying Zhou[a1], Shuyu Zhu[a1], Zhen Huang[b], Jian Chen[c], Jiawei Li[c], Mei Yang[a], Liang Jin[a*], Danqun Huo[a*], Changjun Hou[a,d*].

[a] Key Laboratory for Biorheological Science and Technology of Ministry of Education, Bioengineering College of Chongqing University, Chongqing 400044, PR China

[b] Key Laboratory of Bio-Resource and Eco-environment of Ministry of Education, College of Life Sciences, Sichuan University, SeNA Research Institute and Szostak-CDHT Large Nucleic Acids Institute, Chengdu, 610000, PR China.

[c] Chongqing University Three Gorges Hospital, Chongqing, 404000, PR China

[d] Chongqing Key Laboratory of Bio-perception & Intelligent Information Processing, School of Microelectronics and Communication Engineering, Chongqing University, Chongqing, 400044, PR China

[1] Shiying Zhou and Shuyu Zhu contributed equally to this work

* Corresponding author. Tel/ fax: +862365102507；

E-mail addresses: jinliang4002@126.com (L.Jin), huodq@cqu.edu.cn (D. Huo), houcj@cqu.edu.cn (C. Hou)

2. Material and methods

2.1. Material

All the DNA sequences (Table S1) were synthesized by Sangon (China, Shanghai). EXO I, TE buffer, DEPC-treated H_2O and and PAGE related reagents (Acryl/Bis 30% Solution (29:1), nucleic acid dyes, TBE buffers, loading buffers and DNA marker) were available from Sangon (China, Shanghai). TB green Premix Ex Taq II (Tli RnaseH Plus) was purchased from Takara. The LbCas12a and LwCas13a were purchased from Guangzhou Meige Biological Technology Co., Ltd. HiScribe@T7 Quick High Yield RNA Synthesis Kit, RNase Inhibitor, T4 DNA ligase, Phi29 DNA Polymerase, and NTP mix (25 mM) were supplied by New England Biolabs Inc. (Beijing, China). Tiangen Biotech provided RNase-free H_2O and miRNA purification kit (Beijing, China). Biogrund's human normal serum was used (Chongqing, China).

2.2 Preparation of crRNA

crRNA was synthesized by T7 transcription in vitro using HiScribe@T7 Quick High Yield RNA Synthesis Kit. Firstly, the mixture containing 1 μL of T7 promoter (100 μM), 1μL of crRNA template (100 μM) and 16 μL of RNase-free H_2O were annealed at 95 ℃ for 5 min and cooled down to room temperature slowly. This is followed by a transcription reaction with the addition of 10 μL of NTP mixture (100 mM) and 2 μL of T7 mixture under the conditions of 37 ℃ for 12 h. Afterwards, 2 μL of DNase I were added into the reaction liquid and maintained at 37 ℃ for 2 h to degrade the DNA template. Finally, the product was purified by a miRNA purification kit and quantified with a NanoDrop 2000C (Thermo Fisher). And the obtained crRNA was characterized by gel electrophoresis analysis (Figure S1).

2.3 Target-mediated Rolling Circle Transcription Reaction (TM-RCT)

Different concentration of target was mixed with circular template mixture (50 nM, circular template16 and circular template 18) and denatured for 5 min at 95 ℃, following by linear annealing to 25 ℃. Afterwards, 50 U of T4 DNA ligase (diluted by T4 DNA ligase buffer) were added and reacted at 37 °C for 30 min. Then 40 U of Exo I (diluted by Exo I buffer) were added to continue the reaction for 1 h. Finally,

the reaction was inactivated at 80 °C for 20 min. For rolling circle transcription reaction, 2 μL of RNAPol Reaction Buffer, 2 μL of T7 RNA Polymerase (25 U), 2 μL of RNase Inhibitor (10 U) and 0.5 μL of NTP mix (25 mM) were added to the above solution and was maintained at 37 °C for 150 min to perform TM-RCT, the total volume of amplification system is 20 μL.

2.4 Cas12a/Cas13a-Based Reaction and Fluorescence Measurement

For simultaneous detection, the 20 μL detection mixture was composed of TM-RCT product (4 μL), RNase-free H_2O, buffer 1 (10 x), buffer 2 (10 x), 18-crRNA (100 nM), Cas12a (100 nM), Cas13a (50 nM), RNase Inhibitor (10 U), DNA activator (50 nM), DNA-FQ (500 nM) and RNA-FQ (500 nM). The reagent concentration of the reaction is the final concentration. Subsequently, the reaction solution was placed in a 37 °C vacuum desiccator for 50 min. Finally, 80 μL of ddH_2O was added for collecting the fluorescence intensity using a fluorescence spectrometer (PerkinElmer). An excitation wavelength of 480 nm was used.

For single target detection, the CRISPR/Cas12a system was used to recognized TM-RCT product in presence of HPV16, and the CRISPR/Cas13a system was performed in presence of HPV18. The CRISPR/Cas12a trans-cleavage system was composed of RNase-free H_2O, buffer 1 (10 x), Cas12a (100 nM), DNA activator (50 nM), DNA-FQ (200 nM) and TM-RCT product (2 μL). The CRISPR/Cas12a reaction was incubated at 37 °C for 50 min. For detection of HPV18 target, the CRISPR/Cas13a trans-cleavage reaction system consists of RNase-free H_2O, buffer 2 (10 x), Cas13a (50 nM), 18-crRNA (100 nM), RNA-FQ (500 nM) and TM-RCT product (2 μL). And the CRISPR/Cas13a reaction was maintained at 37 °C for 20 min.

2.5 PAGE analysis

The experimental products were characterized by polyacrylamide gel, and 10% polyacrylamide gel was used, the operation condition was 110 V for 80 min. Agarose gel electrophoresis was used to analyze 18-crRNA transcripts using 1.5 % agarose. The final gel was imaged via a gel imaging system (JENA UVsolo).

2.6 qPCR procedure

The fluorescent quantitative PCR reaction was performed by the TB green Premix Ex Taq II (Tli RnaseH Plus). The reaction system was as follows, 10 μL of TB green Premix Ex Taq II, 0.8 μL of 10 μM Forward Primer, 0.8 μL of 10 μM Reverse Primer, 2 μL of cDNA and 6.4 μL of ddH_2O. The PCR procedure was as follows: 3 min at 95 °C followed by 40 cycles of 95 °C for 5s, 60 °C for 34 s.

2.7 Real sample analysis

The human normal serum was diluted 20 times with RNase-free H_2O. Subsequently, different concentrations of the target are added to the diluted serum. The final concentration of the testing samples was 0.1 nM, 1 nM and 10 nM, respectively. The target with diluted serum was used as test sample for TM-RCT/ Cas12a-Cas13a reaction.

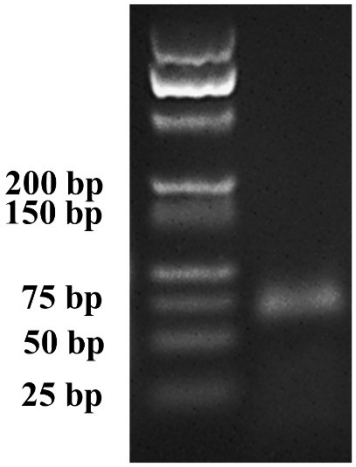

Figure S1 Polyacrylamide gel electrophoresis (PAGE) analysis of 18-crRNA.

Table S1 The DNA sequences used in this study

Name	Sequence (5'-3' direction)
HPV16	TGCAATGTTTCAGGACCCACAG GAGCGACCCAGAAAGTTACCAC
HPV18	GTTCAGGCTGGATTGCGTCGCA AGCCCACCATAGGCCCTCGC
Circular template 16	P- CTGTGGGTCCTATCTACAACAGTAGAAATTACCCTATAGTGAGTC GTATTAATATCTACAACAGTAGAAATTACTGGGTCGCTC
Circular template 18	P- TGCGACGCAATCCACCCTATAGTGAGTCGTATTAATAAAAAAAAA AAAAAAAAAAAAAAAAAAAGCCTATGGTGGGCT
activator-NTS	GCTTGTGGCCG**TTTC**AGGACCCACAGGAGCGACCCAGTAAGATG GGCACCACCCCGGC
activator-TS	GCCGGGGTGGTGCCCATCTTACTGGGTCGCTCCTGTGGGTCCTGA AACGGCCACAAGC
activator'-NTS	GACGACAAAAC**TTTA**ATTAATACGACTCACTATAGGGTAACTGTC TGTGGAATGCTA
activator'-TS	TAGCATTCCACAGACAGTTACCCTATAGTGAGTCGTATTAATTAA AGTTTTGTCGTC
18-crRNA template	GCCTATGGTGGGCTTGCGACGCAATCCAGTTTTAGTCCCCTTCGTT TTTGGGGTAGTCTAAATCCCCTATAGTGAGTCGTATTAATTTC
16-crRNA template	GGGTCGCTCCTGTGGGTCCTATCTACACTTAGTAGAAATTACCCTA TAGTGAGTCGTATTAATTTC
HPV16 (NTS)	GTGGTAACTTTCTGGGTCGCTCCTGTGGGTCCTGAAACATTGCA
T7 promoter	GAAATTAATACGACTCACTATAGGG
HPV16	ATGCACCAAAAGAGAACTGCAATGTTTCAGGACCCACAGGAGCG ACCCAGAAAGTTACCACAGTTATGCACAGAGCTGCAAACAACTAT ACATGATAT
Primer F	GCACCAAAAGAGAACTGCAATG
Primer R	TCATGTATAGTTGTTTGCAGCTCTG
DNA-FQ	HEX-TATTATT-BHQ1
RNA-FQ	FAM-rUrArUrArU-BHQ1
Random 1	TGGCTGGTGGGCAGCGGGTCGCGCGGAGGGCAGCG GCGAG
Random 2	TTTGAGGTGCGTGTTTGTGCCTGTCCTGGGAGAGACCGGCGCACA GAGGAAGAGAATCTCCGCAAG
Random 3	ACTTATCAGCTTAAGGATCATATCACTTCTAATTGATCCTTAAGCT GATAAGT

“P” refers to the phosphate modification.

Optimization of the TM-RCT/Cas12a-Cas13a system

The CRISPR/Cas12a-Cas13a system is the most critical factor to realize simultaneous detection. Several conditions of CRISPR/Cas12a-Cas13a are firstly explored, including buffer component, DNA activator, the ratio of DNA-FQ:RNA-FQ, the ratio of Cas12a:Cas13a and the cleavage time. In the TM-RCT/Cas12a-Cas13a system, two DNA activators can initiate the trans-cleavage activity of Cas12a (Figure S2). This is due that the TM-RCT product, long 16-pre-crRNA, can be pretreated by Cas12a to generate two mature crRNAs. However, the addition of two activators will cause interference signal between CRISPR/Cas systems (Figure S2A), thus, one DNA activator is enough to ensure that the signals do not interfere with each other. In this study, we chose the LbCas12a and LwCas13a for simultaneous detection, which are obtained from Guangzhou Meige Biological Technology. They are performed in buffer 1 and buffer 2, respectively. These buffers have different component. Therefore, four reaction buffers, including buffer 1, buffer 2, NEBbuffer 2.1 and cutsmart buffer) are tested (Figure S3). Experiment results show that only using buffer 1 or buffer 2 has an obvious inhibitory effect on the CRISPR/Cas13a and cause interference peak. NEBbuffer 2.1 also inhibits the activity of Cas13a, and cutsmart buffer has a high background signal. We found that adding buffer1 and buffer 2 at the same time is the most appropriate reaction condition (Figure S3B). Next, we optimized the ratio of DNA-FQ:RNA-FQ, the ratio of Cas12a:Cas13a and the cleavage time of CRISPR/Cas12a-Cas13a. The ideal ratio of DNA-FQ:RNA-FQ is 250 nM:250 nM. The ideal ratio of Cas12a:Cas13a is 100 nM:50 nM. And 50 min is the optimal cleavage time of CRISPR/Cas12a-Cas13a. Excessive time will lead to high background signals (Figure S4A, Figure S4B and Figure S4C).

After optimizing the Cas12a/Cas13a detection parameters, the TM-RCT reaction also plays an important role for the sensitivity of TM-RCT/Cas12a-Cas13a system. Based on the previous research, we optimized the reaction time of TM-RCT. The net

peak value of the two fluorescent signals reaches the maximum in 150 min. Therefore, 150 min is determined as the optimal amplification time (Figure S4D).

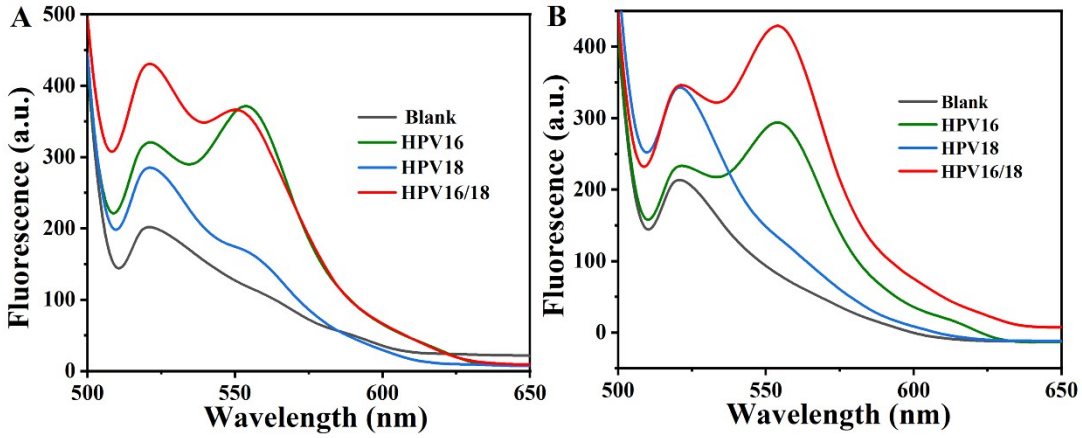

Fig. S2 Measurements of fluorescence emission spectra with two DNA activators (A) and one DNA activator (B) based on the TM-RCT/Cas12a-Cas13a system.

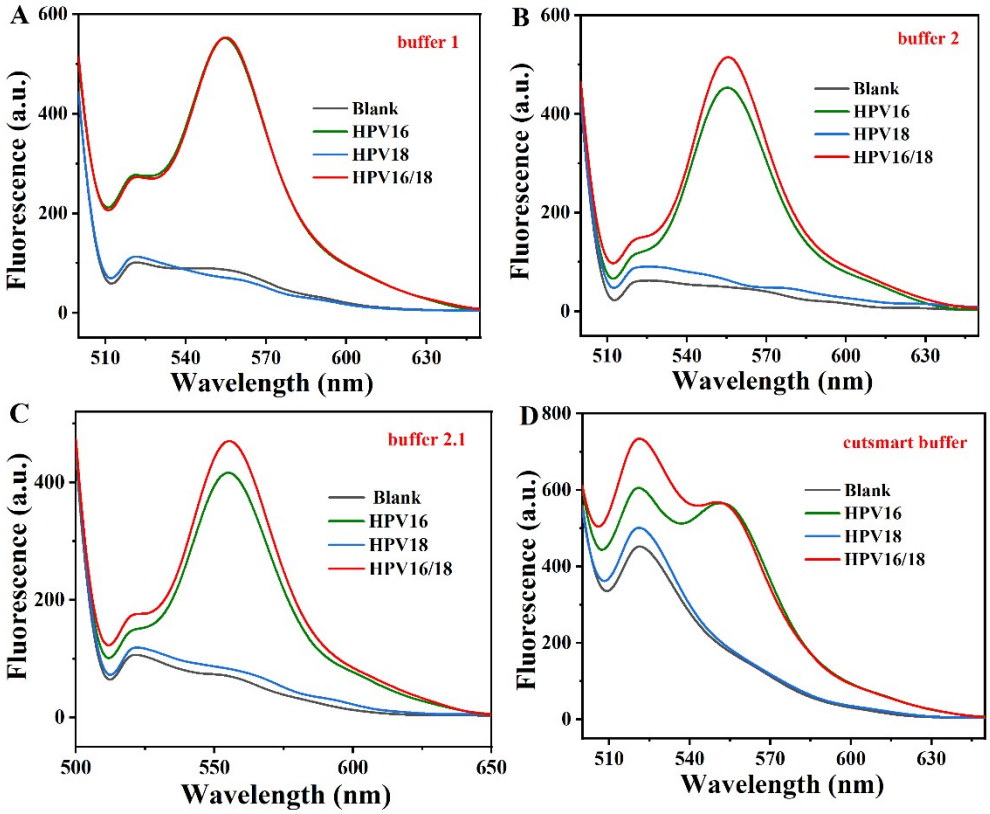

Fig. S3 The fluorescence spectra of the TM-RCT/Cas12a-Cas13a system for simultaneous detection of HPV16 and HPV18 in different buffer.

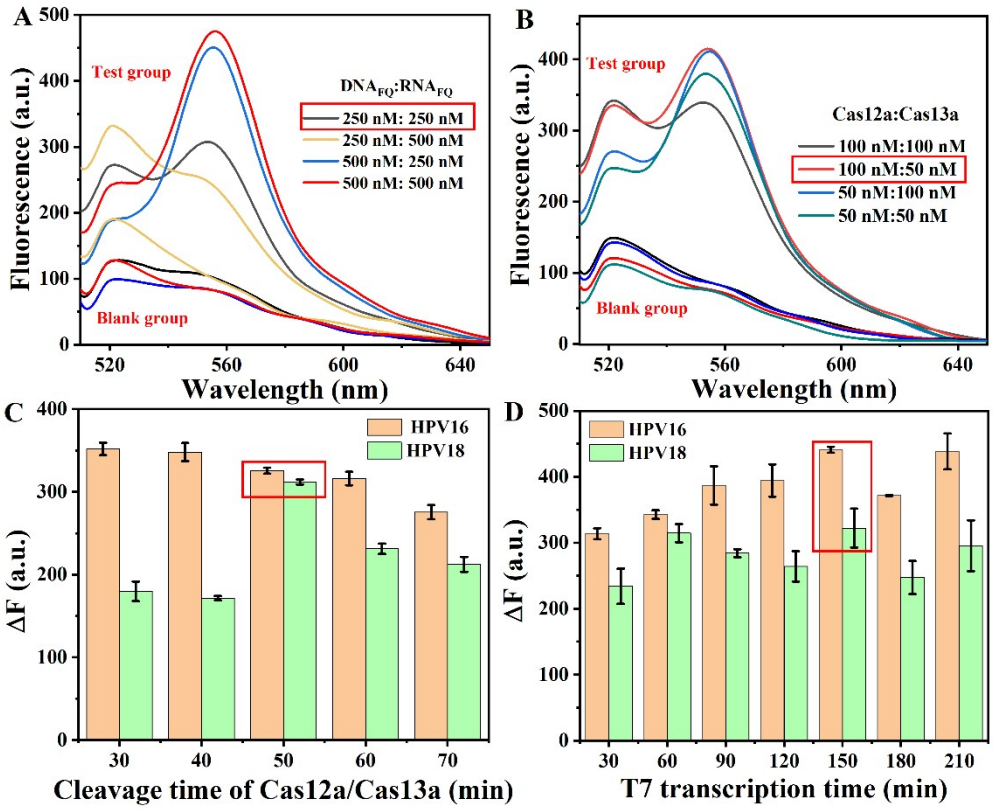

Fig. S4 Optimization experiments of the ratio of DNA-FQ:RNA-FQ, the ratio of Cas12a:Cas13a, the cleavage time of CRISPR/Cas12a-Cas13a and the amplification time of TM-RCT reaction.

We have completed the mutual interference experiment between HPV16 at 555 nm and HPV18 at 520 nm based on TM-RCT/Cas12a-Cas13a system. The concentration of one target is fixed to detect the other, and the fixed concentration is 100 pM. As can be seen from Figure S5, the fluorescence peak with fixed concentration has slight shift, the fluorescence value of the detection target increases with the increase of concentration. This slight shift maybe caused by the two fluorescent probes used in the two systems. Therefore, the efficiency of simultaneous detection is lower than that of independent detection, which is consistent with most literature reports [1,2]. This is because the two fluorescent probes used in the two systems will affect the signal responses and sensitivity [2].

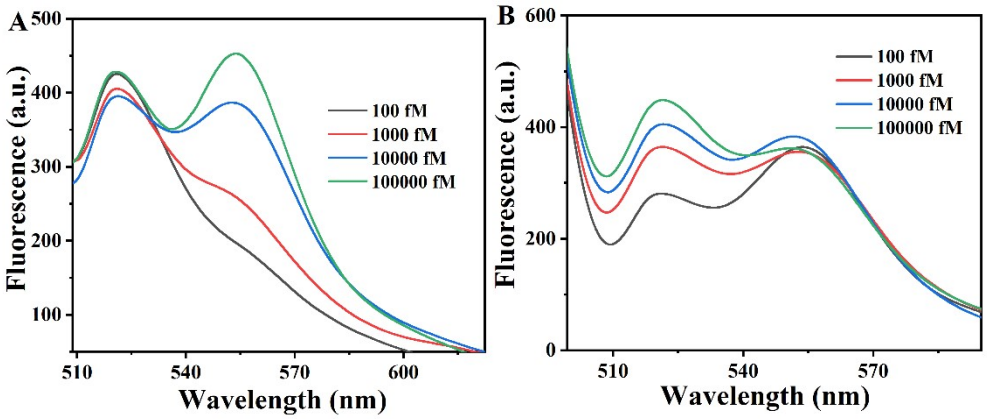

Fig. S5 Mutual interference experiment between HPV16 at 555 nm and HPV18 at 520 nm based on TM-RCT/Cas12a-Cas13a system. The fixed concentration is 100 pM.

[1] G. Cao, J. Dong, X. Chen, P. Lu, Y. Xiong, L. Peng, J. Li, D. Huo, C. Hou, Simultaneous detection of CaMV35S and T-nos utilizing CRISPR/Cas12a and Cas13a with multiplex-PCR (MPT-Cas12a/13a), Chemical Communications 2022,58, 6328-6331.

[2] S. Azzouzi, Z Fredj, A P. F. Turner, M. Ali, W. Mak, Generic Neutravidin Biosensor for Simultaneous Multiplex Detection of MicroRNAs via Electrochemically Encoded Responsive Nanolabels, ACS Sensors, 2019, 4, 2, 326–334.

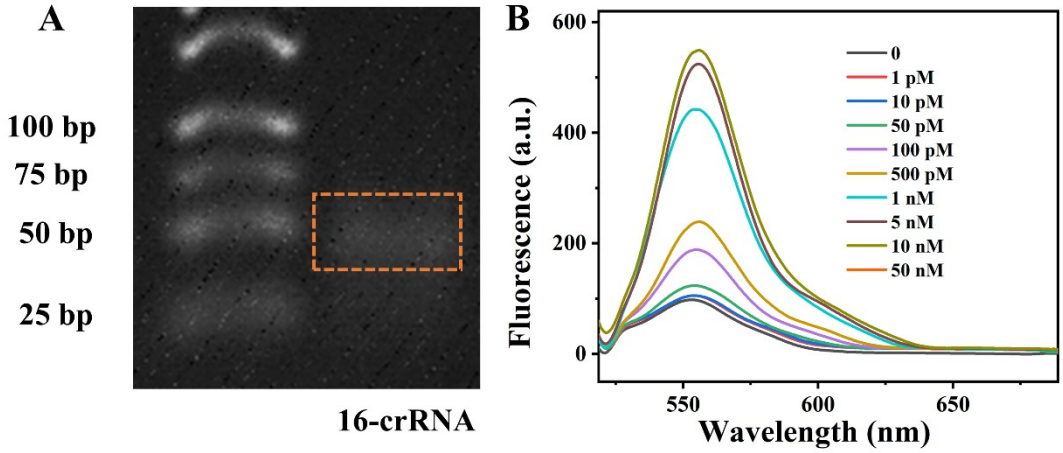

Fig. S6 Polyacrylamide gel electrophoresis (PAGE) analysis of 16-crRNA (A). Fluorescence curves for detection of HPV16 DNA based on CRISPR/Cas12a system (B).

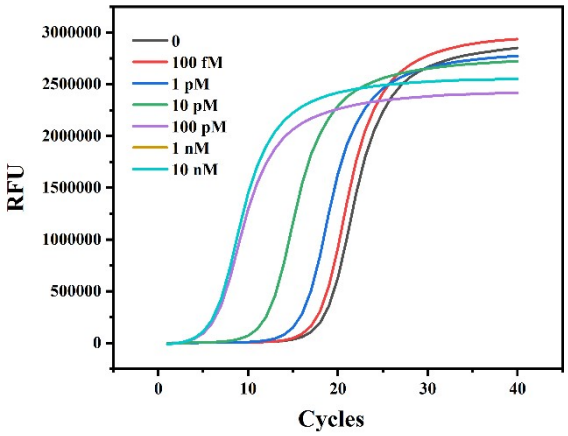

Fig. S7 qPCR results for detection of HPV16 DNA

Table S2 Comparison with other methods for HPV detection

method	Signal amplification	Detection range(M)	LOD(M)	Ref.
Electrochemistry	DNA hybridization with the electron transfer	5×10^{-9} to 2×10^{-8}	2.39×10^{-9}	[1]
Electrochemistry	NH2-IL-rGO/ MWCNT electrodes with redox-active DNA intercalator	8.5×10^{-9} to 1.07×10^{-5}	1.3×10^{-9}	[2]
Electrochemistry	DNA hybridization with anthraquinone-labeled signaling molecules	5×10^{-10} to 1×10^{-7}	1.50×10^{-10} 1.53×10^{-10}	[3]
Electrochemistry	Microfluidic chips with nanostructured films	-	1.05×10^{-11}	[4]
Fluorescence	Exo III assisted fluorescence probe release	5×10^{-10} to 5×10^{-8}	1×10^{-10}	[5]
Fluorescence	T7 Exo assisted target recycling amplification	1×10^{-11} to 4×10^{-9} 2×10^{-11} to 4×10^{-9}	2.3×10^{-12} 5.2×10^{-12}	[6]
Fluorescence	catalytic hairpin assembly (CHA)	1×10^{-11} to 1×10^{-8}	5.7×10^{-12}	[7]
Fluorescence	sunflower seed shells CDs (SCDs)	5×10^{-10} to 1.5×10^{-7}	4.7×10^{-10}	[8]
Fluorescence	water-soluble CdTe quantum dots (QDs)	1×10^{-9} to 5×10^{-8}	2×10^{-10}	[9]

Table S3. The proposed biosensor for HPV16/18 detection in human serum samples

Target added	Detected results (520 nM)	Recovery (%)	Detected results (555 nM)	Recovery (%)
0	-	-		
100 pM	97.72 pM	97.72 %	104.71 pM	104.71 %
1 nM	0.955 nM	95.50 %	0.912 nM	91.20 %
10 nM	9.795 nM	97.95 %	9.749 nM	97.49 %