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Letter

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Fluorescent Probe-Based Lateral Flow Assay for Multiplex Nucleic Acid Detection

Ye Xu,[†,‡,⊥] Yinghua Liu,[§,⊥] Yan Wu,[†] Xiaohu Xia,[†] Yiqun Liao,[‡,∥] and Qingge Li*[,†,‡]

[†]Engineering Research Centre of Molecular Diagnostics, Ministry of Education, School of Life Sciences, Xiamen University, Xiamen 361102, China

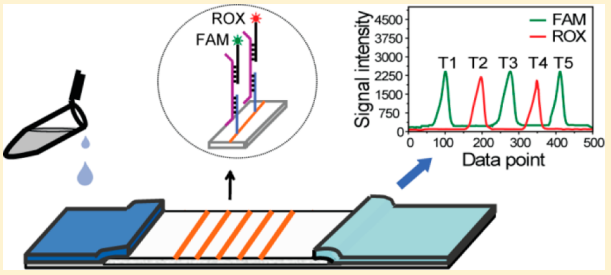
[‡]State Key Laboratory of Molecular Vaccinology and Molecular Diagnostics, Xiamen University, Xiamen 361102, China

[§]Department of Neonatology, Nanjing Medical University Affiliated Suzhou Hospital, Suzhou Municipal Hospital, 26 Daoqian Street, Suzhou, Jiangsu Province 215002, China

[∥]School of Public Health, Xiamen University, Xiamen, Fujian 361102, China

Supporting Information

ABSTRACT: Here we report a rapid, low cost, and disposable dipstick-type DNA biosensor that enables multiplex detection in a single assay. The fluorescent probes labeled with different fluorophores were introduced into the lateral flow nucleic acid testing system. In combination with multiple immobilized probes arranged in an array formant on the membrane, a dual-color fluorescent lateral flow DNA biosensor was developed using a portable fluorescence reader. Up to 13 human papillomavirus types could be detected simultaneously by a single-step operation in less than 30 min after linear-after-the-exponential (LATE)-PCR. The sensitivity was determined to be 10−10^2 copies plasmid DNA/μL. The specificity study showed no cross-reactivity among the 31 different common HPV types. In the clinical validation, 95.3% overall agreement showed very good potential for this method in the clinical application when compared to a commercial kit.



In clinical diagnosis, affordable on-site techniques for the detection of disease biomarkers with high efficiency are urgently needed. However, current techniques often require expensive, sophisticated instruments that may not be available in laboratories with limited resources. Point of care (POC) testing has been emerging as a powerful and effective method to circumvent this problem in recent years.[1,2] Notably, the development of POC nucleic acid biosensors has become increasingly popular due to its great potential in the diagnosis of a variety of diseases.[3−5] Among the various platforms for POC nucleic acid detection, membrane-based lateral flow testing is distinct owing to its ease of use and flexibility for various applications. Lateral flow nucleic acid biosensors based on PCR,[6,7] isothermal amplification,[8,9] or the signal amplification technique[10,11] have emerged in the past few years.

So far, however, most of the current lateral flow-based nucleic acids biosensors rely on the generation or change of colorimetric signal based on the aggregation of colloidal gold nanoparticles. These biosensors are difficult to prepare or produce in large scale due to the complex conjugation procedure involved and are more or less limited to low detection sensitivity because the change or generation of color requires a large amount of particles to aggregate.[12,13] Moreover, these sensors have rather low multiplexing potentiality, limiting the number of analyte to one or two in most applications.[14] Nucleic acid lateral flow assays using up-converting phosphor reporter were also reported.[15,16] Unfortunately, preparation and labeling of these reporters were complicated and multiple steps were needed to accomplish the assay. On the other hand, fluorescence detection is not only very sensitive but it is also capable of multiplex detection. Nevertheless, development of fluorescence-based lateral flow nucleic acid biosensors remains to be achieved.

In this study, we sought to develop a fluorescence-based lateral flow testing with the focus on the multiplex detection. We used human papillomavirus (HPV) as a model analyte, detection of which is critical in diagnosis of cervical cancer or genital warts. We chose those HPV types that are most commonly detected in clinical settings.

The principle of our fluorescence-based lateral flow assay is schematically illustrated in Figure 1. The key feature is that the detection probes, which are fluorophore-labeled, are included in the PCR reaction. When the PCR products were applied to the lateral flow strip where the capture probes are immobilized, driven by the capillary flow, the detection probe, the single-stranded PCR product, and the capture probe will form a sandwich-like hybridization product on the location of the capture probe. The presence of the PCR products was detected on that location using a fluorescence reader. Because capture

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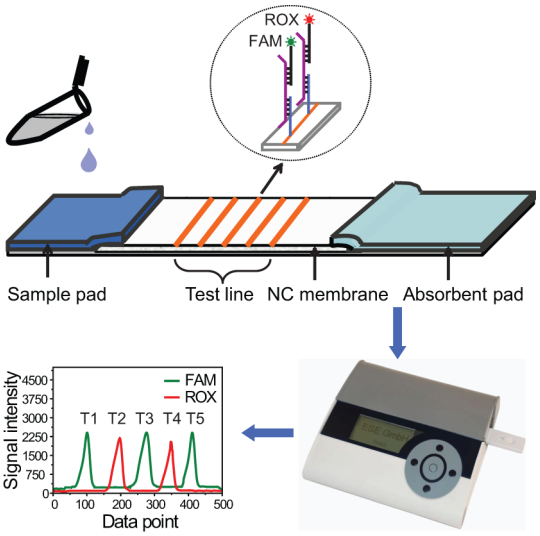

Figure 1. Schematic illustration of the configuration and measurement principle of the fluorescent probe-based nucleic acid lateral flow assay. Along the nitrocellulose membrane, a series of capture probes specific for different targets were immobilized. During the detection process, the targets and the detection probes would migrate through the membrane driven by capillary action. A given target could be specifically captured at the corresponding test line containing capture probe sequence that is complementary to it and the corresponding fluorescence signal would be displayed when scanned in the fluorescence lateral flow reader.

probes can be immobilized on different locations of the strip and the fluorophore-labeled probes can be labeled with different fluorophores, together they can hybridize to many different single-stranded DNA products generated in a multiplexed PCR for detection in a single lateral flow assay.

According to the above principle, we first devised an assay for the detection of four common HPV types, HPV 6, 11, 16, and 18. To avoid potential false negative, a human β-globin (*HBB*) gene was included as the internal positive control (IPC). Thus, the assay for the four HPV types actually involved the detection of five targets in one reaction. A multiplex PCR reaction that could amplify the four HPV types and the IPC was developed. The primers for the four HPV were derived from the consensus GP5+/GP6+ primer pair.[17] One additional primer pair was designed for the amplification of *HBB*. We followed the principle of LATE-PCR, an improved strategy for non-symmetric PCR,[18] to generate an abundance single-stranded PCR products for probe hybridization. For this purpose, an oligonucleotide tail was attached to 5′ termini of each primer. The sequences of the primers are listed in Table S1 in the Supporting Information.

To detect the multiple products generated from the multiplex LATE-PCR, one pair of capture probe and detection probe is designed for each type of amplicon. The capture probe was added with a 3′ terminal poly(dT) spacer in order to ensure high binding efficiency after immobilization.[19] The capture probe was biotinylated and mixed with neutravidin, through which the capture probe is immobilized on the nitrocellulose (NC) membrane. The detection probe was designed to have a melting temperature (T_m) value that is lower than the annealing temperature of the LATE-PCR. The low T_m of detection probes assures no hybridization of the detection probe during LATE-PCR, so that any potential inhibition on the primer elongation and probe degradation by the 5′-exonuclease activity of the Taq DNA polymerase would be avoided. In total, five pairs of probes were designed for the four HPV types and *HBB* (Table S1 in the Supporting Information). Because our customized fluorescence lateral flow reader (ESEQuant Lateral Flow System, Qiagen, Germany) could detect FAM and ROX fluorescence, we assigned the five targets in two channels, i.e., HPV types 6 and 16 and IPC were detected in the FAM channel and HPV types 11 and 18 were detected in the ROX channel.

Details for LATE-PCR conditions, immobilization of the capture probes, fabrication of biosensor, and the detection procedure were provided in the Supporting Information. To prevent the contamination caused by post-PCR operation, dUTP and uracil-DNA glycosylase was used in the LATE-PCR Mix with an added digestion step before PCR. The assay procedure is very simple. Briefly, 5 μL of extracted DNA was added into the 20-μL LATE-PCR reaction. After amplification, 50 μL of the flow buffer was added into the PCR reaction tube, and the whole 75 μL of solution was applied to the sample pad of the strip. After incubation at 45 °C for 30 min, the sensor was inserted to the reader for readout that lasts 30 s for one reading.

To obtain the optimal performance, analytical parameters, including the ratio of the forward and reverse primers, concentration of detection probes, incubation temperature, and lateral flow time, were systematically studied. The results showed that 100 nmol/L GP5+ and 1 μmol/L GP6+ (1:10 primer ratio) gave the highest signal intensity (see Figure S1 in the Supporting Information). To determine the optimal concentration of the detection probes, different probe concentrations (0.8, 1.6, 3.1, 6.2, 12.5, and 25.0 nmol/L) of each HPV type were loaded into the LATE-PCR Mix. A final concentration of 12.5 nmol/L detection probe proved to be optimal for the four HPV types (see Figure S2 in the Supporting Information). The incubation condition was set as 45 °C for 30 min and increased time showed no much fluorescence change within 1 h (see Figure S3 and S4 in the Supporting Information). Other parameters such as lateral flow buffer, type of nitrocellulose membrane, etc. were also investigated (data not shown). Under optimal conditions, one test can be finished within 30 min after LATE-PCR. Because the reading procedure only lasts 30 s, as many as 60 tests can be finished within 1 h after LATE-PCR.

To evaluate the specificity of the assay, 31 common HPV types were tested. These HPV types were standard synthetic plasmids containing the GP5+/GP6+ flanked region of the respective HPV types (HPV 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 68-1, 68-2, 73, 81, 6, 11, 26, 40, 42, 43, 44, 53, 54, 61, 66, 69, 70, 73, and 82) based on the type-specific sequences (http://www.ncbi.nlm.nih.gov). The result showed that none of the 31 HPV types showed cross-reactivity with the established assay (Figure 2). The limit of detection (LOD) was studied for each type. To do so, HPV types 6, 11, 16, and 18 plasmids were each diluted to a series of concentrations of 10^5, 10^4, 10^3, 10^2, and 10^1 copies/μL. The LODs was determined to be 10 copies/μL for HPV type 6, 11, and 16, 10^2 copies/μL for HPV type 18 (Figure 3). This LOD level is comparable to an approved reverse dot blotting kit (GenoArray HPV Test, HybriBio Inc., Chaozhou, China).

We then evaluated the established assay using 157 cervical swab samples, all of which were on-shelf and were previously analyzed by the GenoArray kit. The samples were renumbered and the results of GenoArray were blinded to the person who

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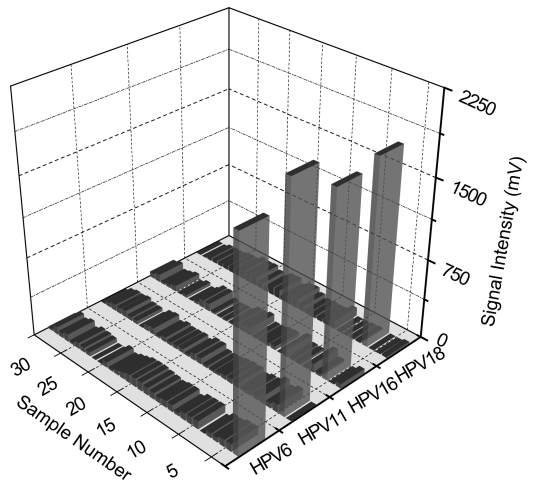

Figure 2. Typical results of cross-reactivity to other HPV genotypes. Samples from 1 to 31 are HPV 6, 11, 16, 18, 26, 31, 33, 35, 39, 40, 42, 43, 44, 45, 51, 52, 53, 54, 56, 58, 59, 61, 66, 68-1, 68-2, 69, 70, 72, 73, 81, and 82, respectively.

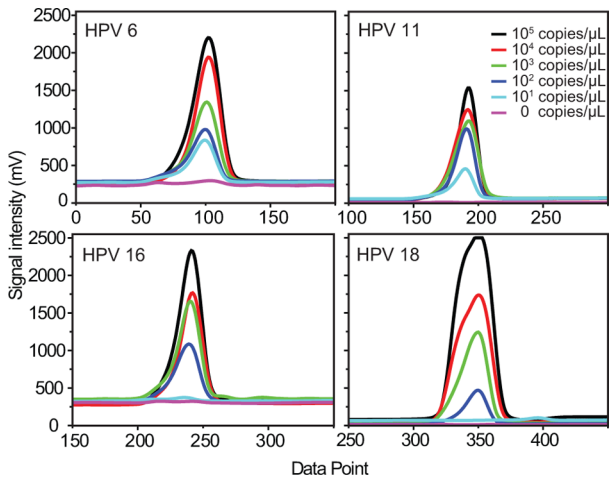

Figure 3. Typical results of the biosensor loaded with different amounts of target plasmid DNA. Black, red, green, dark blue, light blue, and purple curves correspond to 10^5, 10^4, 10^3, 10^2, 10^1, and 0 copies plasmid DNA/μL, respectively.

conducted the detection with our method. The overall agreement between these two methods was 98.1% (154/157). Among the 3 discrepant samples, 1 sample revealed no amplification product by agarose gel electrophoresis, which may derive from low concentration or degradation of the HPV DNA during long-term storage (about 3 months). Of the remaining 2 discrepant samples, DNA sequencing analysis revealed that the result for 1 (50%) sample was concordant with that of our method, the result for 1 (50%) sample agreed with the GenoArray kit. The type-specific concordance between the two methods was shown in Table 1. The concordance between the two methods on the type level was higher than 99%, and the degree of concordance (κ) was more than 0.92. These results demonstrated that the new method is comparable to the GenoArray method in HPV typing.

The above results prompted us to explore the possibility of further increasing the number of targets to be detected in the fluorescence lateral flow assay. To this end, the first step was to increase the number of detection lines on the strip. The detection window of this biosensor is 15 mm in length, which can easily accommodate 7 detection lines. Because the reader has two detection channels, each line position could hold two different capture probes if the paired two detection probes were labeled with different fluorophores. Therefore, in total 14 capture probes could be immobilized on the strip, enabling the same number of targets to be detectable. We chose the most common 13 high-risk HPV types (16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, and 68) currently tested in clinical settings as the targets and *HBB* again as the IPC. An improved multiplex LATE-PCR based on above was established to amplify all the 13 HPV types and *HBB*, and the corresponding 14 pairs of capture probe and detection probes were designed to detect all the single-stranded PCR products. Among the 14 detection probes, 7 probes were labeled with FAM and another 7 were labeled with ROX. After a brief optimization of the reaction conditions and by following the identical working conditions, the established assay could successfully detect all the HPV types when using the plasmid as templates. The LOD was determined to be $10-10^2$ copies/μL. The specificity study showed no cross-reactivity among all the 31 different HPV types. The assay was further subjected to evaluation using a batch of 106 clinical samples. In comparison with the GenoArray kit, the overall agreement between these two methods was 95.3% (101/106), demonstrating the feasibility and accuracy of this assay in clinical use.

In conclusion, our method provides an extremely easy and rapid assay for multiplex nucleic acid detection. It has several advantages over the current nucleic acid testing methods. First, after LATE-PCR, it takes only 30 min to get the detection results without the need of using high-end or expensive machine. Second, the combination of detection probe preloaded in PCR allows post-PCR analysis to be achieved in only one single step. That makes our method significantly more convenient than Southern blot based methods or other reported multiplex nucleic acid lateral flow methods. Third, because all materials required in our system are available in a common molecular laboratory, development of a new assay is straightforward without extra expertise. Finally, we describe here the combined use of fluorescence color and physical position on the nitrocellulose membrane as a virtual two-dimensional label that enables multiplex detection of nucleic acid in a lateral flow assay. It is easy to expand the throughput by just adding more test lines and more fluorescent channels. In

Table 1. Type-Specific Concordance between the GenoArray Method and the Nucleic Acid Lateral Flow Method[a]

HPV type	GA+ NALF+	GA+ NALF−	GA− NALF+	GA− NALF−	total	% agreement	κ [95%]
6	6	1	0	150	157	99.4	0.920 [0.840−1.000]
11	7	0	1	149	157	99.4	0.930 [0.860−1.000]
16	15	1	0	141	157	99.4	0.964 [0.928−1.000]
18	6	0	0	151	157	100	1.000 [1.000−1.000]

[a]GA, GenoArray method; NALF, Nucleic Acid Lateral Flow method.

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combination with advanced portable devices (e.g., battery-operated thermal cycler and multicolor fluorescent reader), our method will perfectly fit the demand for multiplex nucleic acid POC testing in rural or developing areas.

■ ASSOCIATED CONTENT

Supporting Information

Additional information as noted in text, including apparatus and materials, LATE-PCR conditions, fabrication of the lateral flow DNA biosensor, lateral flow assay, and results of the optimization of analytical parameters. This material is available free of charge via the Internet at http://pubs.acs.org.

■ AUTHOR INFORMATION

Corresponding Author

*E-mail: qgli@xmu.edu.cn.

Author Contributions

⊥Y.X. and Y.L. contributed equally to this work.

Notes

The authors declare no competing financial interest.

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