Detection of Deoxyribonucleic Acids on A Membrane Strip

Jui-Chuang Wu, Yai-Hsin Hsu, Dan-Ping Cheng, and Ya-Yuen Cheng

Abstract—**A lateral-flow membrane strip was developed to detect the signal of deoxyribonucleic acids. The signal amplification was demonstrated by the detection of the model sample, biotinlyated Avian-Influenza (AI) genetic sequence, with a sandwich configuration of the target-RCA template complex captured between a RCA (Rolling Circle Amplification) template and streptavidin protein immobilized on a nitrocellulose membrane strip.**

Index Terms—**Avian influenza, biosensors, dna detection, lateral-flow membrane strips, rolling circle amplification**

I. Introduction

The Membrane-based lateral-flow (MBLF) detection is one of the most important tools used for rapid medical diagnoses and public-health research activities. It offers low cost and operational simplicity for end users and also allows untrained personnel to operate in an environment where access to laboratory instrumentation is limited or unavailable [1]-[3].

Many researchers have reported applications of MBLF using immunoassay, for the detections of staphylococcal enterotoxin B [4] and ricin [5] to ensure food safety. The MBLF technology was also combined with multiplex-nested Polymerase Chain Reaction (PCR) to detect various antibodies [6], [7], antigens [8], [9], and allergens [10], [11]. Together these studies have prompted considerable interest in MBLF strips for its potential applications in detection of deoxyribonucleic acids of various pathogens.

This study develops a strategy to directly detect genetic sequences on MBLF strips by an isothermal signal-amplification process. A single-stranded oligonucleotide probe alone is demonstrated how to capture its complementary DNA target on a MBLF strip. The Rolling-Circle-Amplification (RCA) technique was adopted to amplify the signal from that captured DNA target.

The DNA target was designed to add onto a sample pad and subsequently flowed onto its neighboring conjugate pad, on which the target specifically hybridized with the RCA template. Following that, the DNA-RCA template complex migrated ahead onto the membrane and was subjected to a capillary flow in the interstitial space of the membrane.

Continuing along this flow path, the biotinlyated complex was captured by the test line. A Cy5-fluorescence-tagged DNA probe was finally added at the last step to report the detection.

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II. Experimental

A. Materials

The RCA circular template was prepared according to the method as follows. In brief, a 88-mer DNA primer hybridized with two ends of the other 80-mer DNA primer of to produce the RCA circular template. The tail section of the RCA circular template was designed to hybridize with the Avian-Influenza H5, so that the template was able to recognize that H5 sequence. The sequence of the 18-mer detector probe was designed to be identical with the middle segment of the RCA circular template and therefore hybridized with the duplicated strand of the RCA template. The Cy5 fluorescence was labeled at the detector probe to report the final detection result. All of these DNA substances were synthesized by Paily Biotechnology (Taiwan). The T4 enzyme for ligasing Strands A and B into the RCA circular template was purchased from Toyobo (Japan). The Phi29 DNA Polymerase for the RCA process, BSA, and RCA Polymerase buffers for running the RCA reaction were from New England Biolabs (UK).

The genetic template of Avian-Influenza H5 was selected as the model of DNA target. It was synthesized with reference to GeneBank's accession number S68489. The DNA primers of this template for PCR cycles had genetic sequences of biotin-5'gccactc cacaatatacaccc3' (forward) and 5'caaattctctatcctcctttccaa3' (reversed). All these materials were synthesized by Purigo Biotech (Taiwan).

Avian-Influenza H5N1 is a highly infectious pathogens, generally spreading among poultries and birds. The outbreak in 2004 and the later reappearance of H5N1 caused human infections, deaths and anxiety. Several efforts have been made to detect this fatal pathogen [12], [13] and to cultivate effective vaccines to counter it [14], [15].

Nitrocellulose membrane, 5μm AE98, used to load the oligonucleotide probes as the detection base, was purchased from S&S (USA). As other three-dimensional materials, e.g. aerogel that we have developed [16], [17], nitrocellulose provides large vast 3D internal surface to adsorb much more biological analytes [18] than 2D surface of biosensors. Sample pads, 33 Glass, and Conjugate pads, 16S, were also purchased from S&S (USA). Absorbent Pads, CF6, were obtained from Whatman (USA). Plastic backing cards were

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purchased from Adhesives Research (USA).

B. Instruments

A TLC dispenser, Model Linomat 5 (CAMAG, Switzerland), was used to dispense oligonucleotide probe onto the membrane in a tiny amount of 2μl for each dispensing run. A PCR machine, Model PC-320 (Astec, Japan), duplicated the H5 genetic targets. The vertical electrophoresis apparatus, Model MP-300N (Major Science, Taiwan), had a good resolution that was sufficient to verify whether or not the circular RCA template had been successfully prepared. NanoDrop1000 (Thermo Fisher Scientific, USA) was utilized for measuring DNA concentrations. The RCA process was performed in a DO-45 oven (Mandarin Scientific, Taiwan). The fluorescent slide scanner, Model LS IV (Genomic Solutions, Inc., US), was used to excite and read the labeling fluorescent dye at wavelength of 635 nm.

C. Procedure

Preparation and verification of the RCA circular template. A biotinlayted RCA primer was first mixed with a linear RCA template and necessary buffer at 95°C. The mixture was then slowly decreased to 16°C, so that the primer was hybridized with the linear template to form an intermediate circular RCA template. The 3' and 5's ends of the intermediate template were then further ligased together by T4-DNA ligase for 4 hr to produce the final circular RCA template.

Immobilization of streptavidin on membrane. The NC membrane was first affixed onto the plastic backing card. A 0.5cc syringe was loaded onto the TLC dispenser and a 5 cm-long line was printed with 5 μl of the 1.0 mg/ml streptavidin solution.

Assembly of Detection Strips. Twenty-five micro-liter of the circular RCA template was dropped on the conjugate pad and dried at 37°C for 10min. The detection strips were assembled in the order: adhesive membrane, the absorbent pad, the conjugate pad and sample pads, onto the plastic backing card and then cut into 5.9cm×5mm strips.

Preparation of DNA samples. To prepare the single-stranded (*ss*) DNA target of the H5 model, the reverse primer, at concentration of 0.2μM and volume of 2 μl, was mixed with 2 μl of 10μM forward primer. The resultant solution wad then mixed with 1 μl of 50 ng/μl template, 5 μl of 10X PCR buffer, 2.5 μl of 5mM dNTP, and 36.5 μl ddH_2O. After addition of 1 μl of 1.25U/μl Taq, the mixture was quickly loaded into the PCR machine. Each PCR cycle was set as denaturization 95°C for 5.5 min, annealing 50°C for 1 min, and extension 72°C for 1 min. In total 40 cycles were conducted and the final product was purified using the purification kit.

Verification of the RCA reaction on MBLF strips. In addition to verification of the liquid-phase, the RCA template was also tested on a MBLF strip. Eight micro-liter (2 pmole) of the biotinlyated RCA template was mixed with 56 μl of the lateral-flow buffer. The resultant solution was dropped onto the sample pad and passed laterally through the test line with 1X streptavidin printed on the membrane for 5 min.

For the washing step, the lateral-flow wash buffer was dropped directly on the top of the test line for three times, at 1.0 ml each time, to wash off the residual template from the membrane. A 5mm-length section of the membrane was then cut off from the test line. This 5×5 mm square piece was loaded, face-up, into a 200μl PCR tube.

For experimental samples, 50μl of the RCA cocktail was loaded into the tube to immerse the membrane. The PCR tube was allowed rest for 1hr at 37°C. The membrane was then incubated with the Cy5-labeled DNA probe. The membrane pieces finally were washed and stuck on a glass slide to be scanned by the biochip scanner and saved as images.

The LS IV chip scanner was employed to acquire the resulting images in an appropriate laser power and PMT (photomultiplier tube) setting. The excitation/emission wavelength of 635/649 nm was set for sensing Cy5 that was used as the label dye for detecting the DNA probe. The scanning resolution was 10 μm, and the scanning strength was in 35 units.

Detection of AI H5 gene and Image Acquisition The TLC dispenser printed streptavidin and the RCA template on nitrocellulose membrane in 3.64 pmole/mm and the conjugate pad in 0.42 pmole/mm. The biotinlyated-H5 genetic samples were diluted to the desired concentrations. Sixty-four μl of each sample was dropped onto the sample pad, to flow through the conjugate pad, and was hybridized with the RCA template. The resultant H5-RCA template complex then passed through the test line printed with 1X streptavidin on the membrane for 9 min.

III. Result and Discussion

A. Verification of the Circular RCA Template

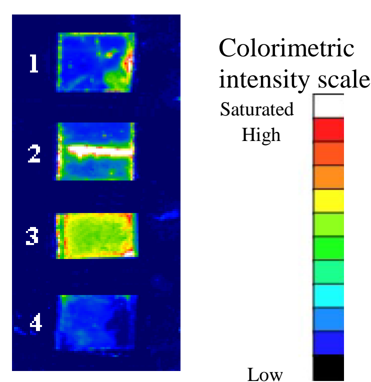

Fig. 1. RCA Reaction on MBLF Strips. Top: Sample 2: RCA Reaction on a static MBLF Strip. Sample 3: RCA Reaction on a shacking MBLF Strip. Samples 1 and 4: negative controls.

To guarantee a valid procedure for the subsequent DNA detection on MBLF strips, the amplification functionality of the purified circular RCA template was verified,. Non-circular RCA templates were expected to waste reagents and significantly lower the efficiency of signal amplification. From the gel electrophoresis, the amplified RCA product was clogged at the top of gel grooves. Its size was no doubt too large to pass through 2% agarose pores, which normally provide a good resolution for sizes of 0.2-1kb DNA.

B. RCA Reaction on MBLF Strips

As shown in Fig. 1, the RCA reaction was performed on MBLF strips. Sample 2 showed an intense test signal, indicating that a MBLF strip was capable of carrying out a RCA signal amplification. The strong background on Sample 3 was attributable to a violent contact between the RCA cocktail and the membrane. The cocktail components stuck inside the membrane matrix due to a rotational shaking. Thus, the next-added Cy5-label probe also stuck on the membrane. Two negative-control strips validated this analysis. Sample 1, which allowed the RCA template to flow through the test line but no RCA reaction occurred, showed a clean background as did Sample 4, which only allowed a plain buffer to flow through the strip.

C. Optimization of Volume and Incubation Time of the Cy-5 Labeled Probe

The addition volume and incubation time for the Cy5-label probe were optimized to obtain a minimum reagent consumption and background but in a maximum signal intensity. As shown in Fig. 2 (a), the sample added with 15 µl of DNA probe received the best result. Therefore 15 µl was chosen for the rest of experiment.

The incubation time for the Cy5-labeled DNA probe was also optimized in order to obtain the best detection result. Fifteen micro-liter of the probe was added to each sample followed a wait of 1, 5, 7 or 9 min. As shown in Fig. 2 (b), the color development was not complete at 3 min. As the incubation time proceeded to 5 min, it started to appear a good signal intensity. The sample incubated for 7 min had a signal interrupt in the middle section because of uneven flow, while the sample incubated for 9 min has a perfectly shaped line and an intense signal. Therefore an incubation time of 9 min for the DNA probe was chosen for the rest of experiment.

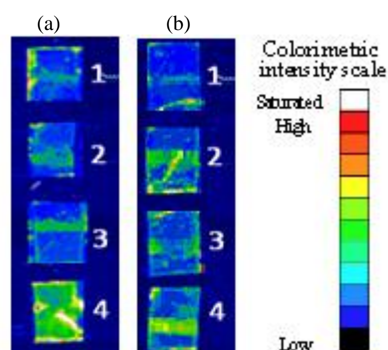

Fig. 2. Optimization of the addition volume and incubation time of the Cy5-label DNA probe. (a) Signals on membrane with volumes of the DNA probe of 5, 10, 15 and 25 µl for Samples 1 to 4, respectively. (b) Time for Samples 1 to 4 was 1, 5, 7 and 9 minutes. The best result was 15 µl of probe incubated for 9 minutes.

D. Optimization of Dispensing Amount of RCA Template

Fig. 3 shows the optimization results of the RCA template's dispensing amount on the conjugate pad. The detection signal appeared on the strip but in a faint intensity, as 0.83 pmole of the template was loaded. As the amount was increased to 1.66 pmole, the signal apparently was intensified. The strong background for 3.32-pmole sample indicated that too much template was loaded.

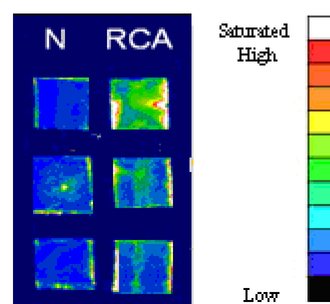

Fig. 3. Optimization of the Dispensing Amount for the RCA Template. Strips N: the negative control, loading plain lateral-flow buffer for the RCA reaction; Strips RCA: experimental strips with amount of RCA template from the top, 3.32, 1.66, and 0.83 pmole. Dispensing amount of 1.66 pmole received the best result.

E. Detection of H5 AI on MBLF Strips

Fig. 4 shows the images of MBLF strips obtained after performing a series of detections on different amounts of the H5-AI target is in the range of 0.2-18.6 pmole. Compared with the detection limit of 6.2 pmole H5 analyte on the traditional gel electrophoresis (data not shown), the MBLF tests still can read the detection at 0.8 pmole.

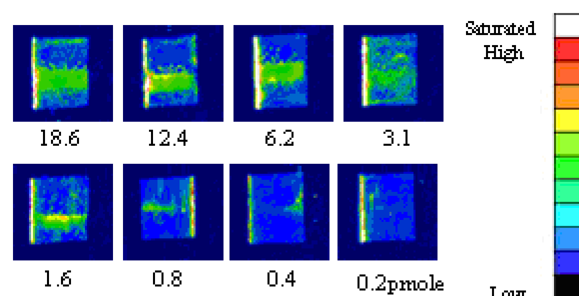

Fig. 4. Detection of H5 AI Gene on the MBLF Tests. The detection limit on the MBLF Tests is shown at 0.8 pmole.

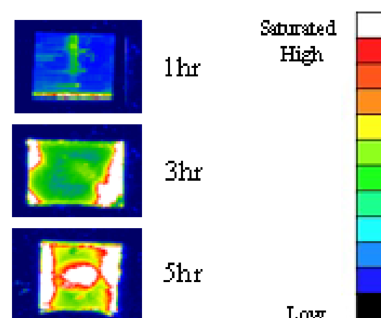

Fig. 5. Influence of Incubation Time on the Backgrounds. The incubation time was set at 3, 5, 7, 24 and 30 hr. All strips showed strong backgrounds and the signals couldn't be recognized from the backgrounds.

To confirm if the signal intensity could be improved by a longer incubation time, an experiment was conducted by extending 1 hr to 3 and 5 hr. As shown in Fig. 5, all strips received very strong backgrounds due to the residual Cy5-labeled DNA probe on the nitrocellulose membrane. For incubation time longer than 1 hr, the DNA probe exhibited stable adhesion with nitrocellulose molecules and any trials to wash it off from the membrane were in vain.

IV. Conclusion

This study successfully developed an isothermal

DNA-amplification process on MBLF strips in necessary operation conditions. It demonstrates how to proceed the detection procedure by a simple isothermal incubator for the recognition of a DNA and amplify the detection signal after sequence on the MBLF strips is complete. With 2.3 pmole of RCA circular template loaded on the conjugate pad, 0.8 pmole of H5 Avian-Influenza (AI) genetic sequence could be detected in 1hr, compared with the detection limit of 6.2 pmole on a traditional gel electrophoresis. Secondly, this methodology utilized less equipment than traditional PCR technology. A mini outdoor isothermal incubator can already perform the application. In addition, this study proved that the signal still can be remained on the membrane. The traditional PCR normally amplifies a DNA template to liquid-phase replicates, such that it no longer holds the signals on the immobilized spot. This detection approach also provides a great potential to incorporate an in-parallel screening assay for a mixture of genetic samples, using multiple DNA probes arrayed on the MBLF strips. With such a DNA-probe array, the MBLF device is capable of simultaneously identifying various pathogens' genetic signatures on the same membrane panel.

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