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Rapid nucleic acid detection of Zaire ebolavirus on paper fluidics

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We described a novel system of paper fluidics, in which isothermal nucleic acid amplification and detection are performed. This paper system made the nucleic acid test simpler, easier to operate, cheap, and provided the performance of point-of-care testing. This system was used to detect the Ebola virus rapidly with high specificity and sensitivity.

The single-stranded RNA Zaire ebolavirus is a fatal infectious disease virus, leading to Ebola hemorrhagic fever in humans and primates, with has an extremely high mortality rate (90%). [1] This pathogen has already caused widespread deaths and heavy economic losses in Western African countries. It is important to develop a fast, cheap and efficient method for the point-of-care testing (POCT) of pathogens in West African countries and regions.

The gold immuno-chromatographic assay, based on the specific binding of antigen/antibody, is one of the successful tests for the rapid and point-of-care testing (POCT) of pathogens. This technology is suitable for poor countries and regions; [2] However, the sensitivity of the assay is limited. Nucleic acid-based tests, such as PCR assays [3], microarray [4] and Next generation sequencing (NGS) [5, 6] , are mo sensitive and specific than antigen- and antibody-based assays. However, the sophisticated equipment and skilled technicians required limit their wide applications in the rapid and point-of-care detection of pathogens. Therefore, it i important to develop a novel detection system that is as convenient as the gold immuno-chromatographic assay and as sensitive as the nucleic acid tests. In this work, we describe a simple paper fluidics method for the nucleic acid amplification and validated by its application to the rapid detection of Zaire ebolavirus.

We first used a nucleic acid biomarker rather than a protein biomarker. This is because nucleic acid-based assays are much more sensitive and specific than protein-based assays. [7, 8] For the Zaire ebolavirus (ZEBOV), we re-evaluated the potential target regions by the alignment with other species of EBOV (Sudan ebolavirus, Reston ebolavirus and Ivory Coast ebolavirus). We chose a fragment of the partial trailer region from position 18,301 to 18,610 (ZEBOV strain Mayinga, GenBank Accession no. AF086833). [9, 10] This nucleic acid fragment is conserved among ZEBOVs and dissimilar with other types of Ebola virus (Detail nucleotic sequence could be found is the reference 9, 10). We made use of the artificial target RNA fragment of Zaire ebolavir s (ZEBOV) (Takara Co. Ltd., Dalian, China) as the main material, while the genetically engineered Escherichia Co containing a plasmid harbouring the target gene fragmer ,

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were applied as the control. (Supplied by Sangon Co. Ltd., Shanghai, China). The positive amplification of this nucleic acid fragment indicate the presence of the Zaire ebolavirus in the sample.

This nucleic biomarker could be detected by various methods, including polymerase chain reaction (PCR), gene chips, nucleotide sequencing and other nano-material-based assays [11, 12]. However, these methods demand sophisticated equipment and skilled technicians, which make them unsuitable for the point-of-care detection of ZEBOV, especially in poor countries/regions. In this work, we used the technology of loop-mediated isothermal amplification (LAMP), integrated with the paper microfluidics.[13, 14] LAMP assay was invented by Notomi and has been widely used to detect various pathogens. However, LAMP assays have been typically performed in PCR tubes using precision pipetting devices in the liquid state, which we believe are not suitable for POCT applications.

The technology of paper microfluidics was developed by Prof. George Whitesides (Harvard University), and has been used in various applications, such as immunoassay, blood/urine analysis and fast metal tests, demonstrating its utility in POCT.[15, 16] However, this technology has seldom been used for nucleic acid assays, especially nucleic acid amplification and detection. We believe that it is essential to perform the nucleic acid amplification on the paper fluidics. Dry chemistry and solid phase operation can be achieved by paper fluidics. In theory, dry chemical technology is more suitable for POCT assays. Additionally: (1) compared with other typical nucleic acid detection methods, such as PCR tubes and glass gene chip, paper-fluidics have significant advantages of the cost and production scale; (2) after use, paper-fluidics, bio-waste, can be destroyed easily and safely by burning; and (3) the potential for sample pretreatment. There are many commercial paper-based products for sample pretreatment and DNA extraction, such as the FTA card, indicating the potential integration of this kind of paper-fluidics.

In this study, we made use of a glass fibre membrane (GF/F, GE Co. Ltd.) to develop the paper fluidics for nucleic acid amplification and detection. The computer-directed X-Y knife cutter was used to design and cut the glass fibre material into the various paper fluidics. Briefly, a piece of glass fibre membrane (Whatman GF/F) was pressed tightly with the adhesive surface of the cutting mat (this was provided along with the cutting machine). The paper fluidic pattern was pre-designed using the software, while the X-Y knife cutter (Cricut Expression® 2, Provo Craft & Novelty, Inc., USA) was used to cut the glass fibre membrane into a predesigned pattern, controlled by the software. We then applied t lamination technology (the laminating film was purchas from Shanghai Goldbio Tech Co., Ltd) to seal the paper fluidics to form the integral micro-reactor.[17, 18]

RT-LAMP reaction reagents were pre-coated and freeze dried to form a functional paper fluidics to amplify/detect the conserved nucleic acid target of ZEBOV. The RT-LAMP reaction was performed according to previous studies, with minor modification. The reaction volume was 10 μL, which contained 1×ThermoPol Buffer (New England Biolabs Inc., USA), 8.0 mM MgSO4, 0.8 M Betaine (Sigma, Germany), 1.0 mM dNTPs (Invitrogen, USA), 0.2 μM each of the outer primer F3/B3, 1.6 μM each of inner primer (FIP/BIP:), 50 nM SYBR Green i and 0.32U/μL of Bst polymerase (Large Fragment; New England Biolabs Inc., USA) and 5U AMV Reverse Transcriptase (Invitrogen); These reagents were all pre-coated on the paper matrix by freeze drying. [19, 20]

A 10-μL volume of sample was introduced via the inlet of the paper fluidics and allowed to fully flow into the microreactor by the capillary force of the paper fibers. Then, the inlet and outlet were tightly sealed with thermosol to form an integral micro-chamber for LAMP amplification and detection. We incubated the whole paper fluidics at 63 °C for 45 min in a water bath. The results were observed under UV light.

The Calcein-Mn^{2+}-$P_2O_7^{4-}$fluorescence system (a kind of fluorescence resonance energy transfer system, FRET) was used to detect amplification product. In this FRET signal system, the pyrophosphate generated during the nucleic acid amplification displaces the Mn^{2+} from the calcein, resulting in emission of green fluorescence that could be detected by UV light (360 nm). We believe this is the first study to perform direct nucleic acid amplification and integration of the LAMP and the Calcein-Mn^{2+}-$P_2O_7^{4-}$fluorescence system in paper fluidics for the rapid detection of the Zaire ebolavirus.

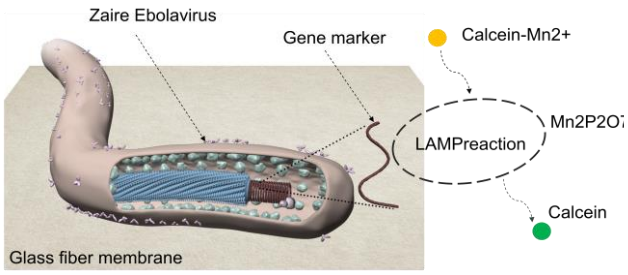

Figure 1. Schematics of the Zaire ebolavirus and its amplification and detection on paper fluidics.

We developed three types of LAMP paper fluidics for the rapid amplification and detection of the conserved nucleic acid fragment of ZEBOV, namely Paper-LAMP fluidics, Paper-RT-LAMP fluidics and Paper-mLAMP fluidics. Each one has its unique features. These functional paper fluidic devices could all be easily designed and fabricated using the computer-directed X-Y cutting technology, while LAMP coupled with the calcein-Mn^{2+}-$P_2O_7^{4-}$ fluorescence FRET system was integrated in this paper fluidic system. [21]

As shown in the **Figure 2 (A)**, Paper-LAMP could amplify the DNA sample directly in the paper matrix, and green fluorescence displayed for the positive signal, while the Paper-RT-LAMP fluidics contains the AMV transcriptase on the paper matrix and could amplify and detect RNA targets directly. In this work, we used an artificial ZEBOVA RNA to certify this paper fluidic. This type of LAMP fluidics could be widely used in the detection of RNA-pathogens.

Paper-mLAMP fluidic was designed to amplify and detect multiple targets (DNA/RNA) simultaneously. This type of paper fluidics would be much more stable and accurate, because of the inner-control in the paper device. We first pre-coated ZEBOV primer, positive LAMP primers and negative control in the three different paper strips. Inclusion of the positive and negative controls made the whole detection result much more stable and reliable, and indicated the integrity of the RNA of the sample **(Figure 2C)**.

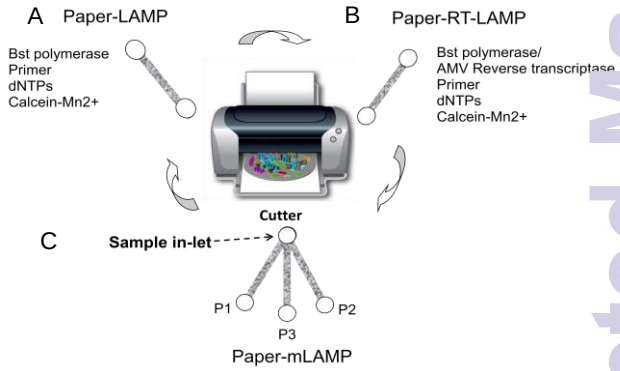

Figure 2. Schematics of the three types of LAMP-paper fluidics

A.Paper-LAMP for the DNA amplification and detection; B. Paper-RT-LAMP for the RNA amplification and detection; C.Paper-mLAMP for the multiple nucleic acid fragment amplification and detection.

We made a comprehensive evaluation of the sensitivity and specificity for the m-paper fluidics of Zaire ebolavirus. Three different primers were pre-coated on the paper matrix 1, 2 and 3, respectively. The serial dilutions of the artificial RNA of ZEBOV (10^{-3} to 10^{-7}) were prepared and applied as templates (original RNA concentration was 10 ng/μL) to demonstrate the sensitivity of the m-paper fluidics. As shown in the **Figure 3 (A)**. The detection limit of the assay was 10 fg RNA, which was comparable to the typical LAMP assay and surpass than the common PCR assay.

Although, we could not get the real clinical samples of ZEBOV to evaluate the specificity and practical application of this paper fluidics for ZEBOV, in this study, we sythesized

three different artificial types of ebolavirus, namely Sudan ebolavirus, Reston ebolavirus and Ivory Coast ebolavirus, to verify the specificity of this novel assay for Zaire ebolavirus. Meanwhile, because of the highly similarity of the genome between the common influenza virus and ebolavirus, additional experiments have been performed to verify the specificity of the assay. All of these experiment results demonstrated the good specificity **(Figure 3 B)**, only Zaire ebolavirus could lead to the positive LAMP amplification on the paper fluidics.

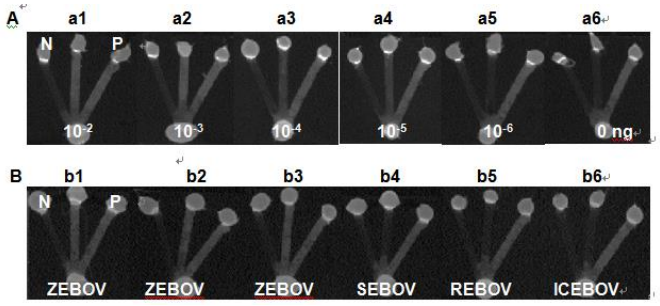

Figure 3. Sensitivity and specificity of isothermal nucleic acid amplification on paper fluidics

a1~a6: The concentration of the artificial RNA of ZEBOV was from 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}, 10^{-6}, 0 ng/μL.

b1~b3: Three repeat of the LAMP paper for the Zaire ebolavirus; b4~b6, Artificial nucleic acid fragment of the Sudan ebolavirus, Reston ebolavirus an Ivory Coast ebolavirus.

In conclusion, we designed a type of paper fluidics for the rapid nucleic acid amplification and signal detection. We used this technique for the point-of-care detection of Zaire ebolavirus (ZEBOV). This type of paper fluidics is capable for the high industrialized production, and could be expanded easily to the analysis of other pathogens or species.

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