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A paper-based platform for detection of viral RNA†

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Abstract

Viral detection presents a host of challenges for even the most sensitive analytical techniques, and the complexity of common detection platforms typically preclude portability. With these considerations in mind, we designed a paper microzone plate-based virus detection system for the detection of viral genetic material that can be performed with simple instruments. The sensing system can detect viral cDNA reverse-transcribed from total RNA extraction by utilizing a biotinylated capture probe and an Alexa Fluor® 647-labeled reporter probe. The biotinylated capture probe was linked to the paper surface *via* NeutrAvidin® that was physically adsorbed on the paper. After addition of reverse-transcribed sample and reporter probe in sequence, the reverse-transcribed target captured the reporter probe and tethered it to the capture probe in a bridged format. Fluorescence intensity was imaged using a Western blot imaging system, and higher target concentration was visible by the increased emission intensity from Alexa Fluor® 647. By utilizing paper, this detection setup could also serve as a sample concentration method *via* evaporation, which could remarkably lower the detection limit if needed. This detection platform used Epstein-Barr virus (EBV) RNA as a proof-of-concept by sensing cDNA resulting from reverse transcription and can be further expanded as a general method for other pathogens. EBV is a well-known human tumor virus, which has also recently been linked to the development of cervical cancer. The assay was accomplished within two hours including the room-temperature RNA extraction and reverse transcription steps. Also, this paper microzone plate-based platform can potentially be applicable for the development of point-of-care (POC) detection kits or devices due to its robust design, convenient interface, and easy portability. The experiment could be stopped after each step, and continued at a later time. The shelf-life of the modified paper plate setup was at least 3 months without a discernible change in signal, and the result from day 1 could be read at 3 months – both of which are important criteria for POC analytical testing tools, especially in resource-poor settings. All of the required assay steps could potentially be performed without any significant equipment using inexpensive paper microzone plates, which will be ideal for further development of POC testing devices. Although, this platform is not at the stage where it can be directly used in a point-of-care setting, it does have fundamental characteristics such as a stable

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platform, a simple detection method, and relatively common reagents that align closely with a POC system.

Introduction

Rapid virus detection with high sensitivity and selectivity is critical in many fields such as food production, disease diagnosis, and environmental monitoring.^{1,2} As reported by the WHO, infectious disease was the leading cause of death in low-income countries.³ Also, it was the major cause of deaths in children below age 5 worldwide in 2015.⁴ The recent viral outbreaks of Ebola and Zika have also brought the attention of the scientific community to the need for on-site, fast, and easy detection of pathogens. To prevent the transmission of infectious disease, early diagnosis and treatment is very important. However, high-quality diagnostic tests are typically unaffordable or inaccessible to people in underdeveloped countries, and the inexpensive diagnostic methods available in these areas cannot provide enough information.⁵ Therefore, a cheap, portable, high-quality virus detection method, which can be used in resource-limited environments, is in high demand. In that respect, the emphasis is now on adapting common assay principles to platforms that are portable and cheap.

Early diagnosis of pathogen by detecting its nucleic acid is a well-accepted method for various reasons, including the high selectivity that comes from hybridization, the low cost and high stability of antisense capture and/or detection nucleic acids relative to antibodies, and the large number of modifications that are commercially available for easy labeling or conjugation of oligonucleotide probes. Common detection methods for viral nucleic acids include PCR,^{6–8} isothermal amplification methods,^{9–11} electrochemical hybridization,^{12–14} surface plasmon resonance (SPR),¹⁵ piezoelectric sensors,¹⁶ optical sensors, and cantilever devices,¹⁷ but these are all typically performed using expensive instruments and/or elaborate laboratory set-ups. As an alternative approach for virus detection, we decided to perform viral nucleic acid detection on a paper-based device due to inherent advantages of this format. It is inexpensive, easily disposable (biodegradable and combustible), conducive to native biomolecule conformations, and a good sample and reagent carrier. These benefits make paper a suitable material for detection in remote environments or in resource-poor settings. PCR for DNA detection from dried blood spots is a well-developed technique.^{18,19} However, practical paper microfluidic devices did not become viable platforms until an easy fabrication method was published in 2007.²⁰ Several paper-based designs have been published recently for nucleic acid detection,^{21–26} and many good reviews describe the importance and variety of paper-based detection methods.^{27–31} Surprisingly, only a few virus detection methods have been applied to paper-based substrates.^{32–37} A recently published paper by the Whitesides group describes the design of a “paper machine” device that integrates sample preparation, *E. coli* DNA amplification (loop-mediated isothermal amplification), and signal detection using a hand-held UV source and camera phone.³⁸ This report is an important milestone since it describes a complete paper-based device for DNA detection. The paper 96-well plate used in our study was termed a “paper microzone plate” and was also designed by the Whitesides group at Harvard University.³⁹ From only a drop of blood, the authors were able to test liver function⁴⁰ or glucose concentration⁴¹ with two

different paper-based setups (colorimetric and electrochemical), which proved that paper could serve as an alternative or even superior material for microfluidic devices. Multiple reports have recently been published using paper microzone for the detection of small molecules;^{42–44} however, we have been unable to find any examples of nucleic acid-based viral detection.

The model viral target we tested was Epstein-Barr virus (EBV), also named human herpesvirus-4 (HHV4). EBV is a double-stranded DNA virus which is transmissible *via* nasopharyngeal secretions. More than 90% of people are infected, often in childhood, and usually experience few if any symptoms or complications.⁴⁵ However, it has about 50% chance of causing infectious mononucleosis (not fatal) if contracted in adolescence.⁴⁶ It is also associated with nasopharyngeal carcinoma.^{45,47} EBV has been linked to progression of cervical cancer and has been found in cervical tissues, especially in high-grade lesions. EBV typically targets B lymphocytes due to strong receptor homology for EBV surface antigens, but other immune cells as well as unrelated cell types can be infected due to the complex surface antigen complement on herpes viruses.⁴⁸ Reported targets for EBV detection include antigen and RNA (Epstein Barr encoded RNAs (EBER)), but RNA presents a more lucrative target given that, on average, there are 10^7 copies of EBER (EBER-1 and EBER-2) present in one latently infected cell.^{49,50} These two RNAs are small, non-coding RNAs which are usually bound to nuclear proteins.⁴⁷

In this manuscript, we outline a viral nucleic acid-based assay that relies on DNA hybridization to specific, surface-immobilized capture probes on a paper substrate, allowing our system to be utilized as a general method for viral nucleic acid detection with the possibility of multiplexing. Briefly, as shown in Fig. 1, the first step of the assay involves lysis of EBV-carrying cells or blood lymphocytes and reverse-transcription of RNA to cDNA at room temperature within 10 min. Next, single-stranded cDNA target was generated by hydrolyzing the RNA template in a boiling water bath for 10 min under basic conditions. After the boiling step, the single-stranded cDNA target was captured on a paper microzone surface by DNA hybridization. To achieve this, a biotinylated-capture probe was immobilized on a paper surface that was previously coated with physically-absorbed NeutrAvidin® (NA). The cDNA was detected with Alexa Fluor® 647-labeled reporter probes, and the fluorescent result was imaged with a LI-COR instrument and analyzed with ImageJ.

Material and methods

Materials

NeutrAvidin® (NA), dNTPs, Alexa Fluor® 647-oligo, herring sperm, and RNaseOUT were from Invitrogen (Grand Island, NY). RPMI media, fetal bovine serum, biotinylated capture probe, and unmodified oligo were purchased from Sigma-Aldrich (St Louis, MO). StartingBlock T-20 blocking buffer was from Pierce (Rockford, IL). The Raji B cell line was purchased from American Type Culture Collection (Manassas, VA). The RNeasy Mini Kit was from Qiagen (Valencia, CA). M-MLV reverse transcriptase was obtained from Epicentre (Madison, WI), and Whatman #1 cellulose paper was purchased from Whatman

(Piscataway, NJ). Human whole blood was from Zen-Bio, Inc. (Research Triangle Park, NC). Ficoll-Paque PLUS solution was purchased from GE Healthcare (Waukesha, WI).

Cell preparation and RNA extraction

Raji B cells were cultured at 37 °C, 5% CO₂ in RPMI media with 10% fetal bovine serum. After centrifugation, total RNA was extracted using an RNeasy Mini Kit based on the Qiagen protocol. Briefly, approximately 1×10^7 cells were lysed in 0.5 mL buffer RLT. Then, a 0.5 mL aliquot of 70% ethanol was added to the mixture. The solution was transferred to a filter column, and RNA was collected by centrifuging for 15 s. The filter was washed with 700 μ L of buffer RW1 and centrifuged for 15 s. Then, a 500 μ L aliquot of buffer RPE was added to the column, and the column was centrifuged for 2 min. The empty column was centrifuged for 1 min before adding 30 μ L of 60 °C RNase-free water. The filter was incubated for an additional 1 min before centrifuging for 1 min. All centrifuge steps were performed at room temperature and 10 000g. The concentration of total RNA was determined using a GE NanoVue Plus spectrophotometer (Hollywood, FL).

Single-strand cDNA synthesis

Reverse transcription was performed according to the Epicentre M-MLV protocol. Briefly, extracted total RNA was mixed with 0.5 nM RT primer, 2 μ L of 10 $\times$ buffer, 10 μ M DTT, 0.5 μ M of each dNTP, 100 U M-MLV reverse transcriptase, 40 U RNaseOUT, and RNase-free water to a total volume of 20 μ L. The solution was incubated at room temperature for 10 min, followed by RNA hydrolysis in a boiling water bath for 10 min with 0.33 M NaOH. After neutralization with HCl and the addition of buffer, the single stranded cDNA was used for detection.

Design and use of paper microzones for detection of synthesized oligo and cDNA reverse transcribed from the extracted RNA from Raji B cells

96-well paper microzones were fabricated according to previous literature protocols.^{39,51} Briefly, the 96-well pattern was designed with Adobe Illustrator and printed on the filter paper surface using a Xerox ColorQube 8570 solid ink printer (Xerox; Norwalk, CT). The wax was melted by incubating for 1 min at 110 °C. To assemble the paper capture construct, a 4 μ L aliquot of 4.2 μ M (0.25 mg mL⁻¹) NA was allowed to adsorb for 30 min in each fabricated reaction zone. After washing once with PBS buffer (10 mM sodium phosphate, 150 mM sodium chloride pH 7.2), a 4 μ L aliquot of 4 μ M biotinylated capture probe (b-Tar, Table 1) was incubated in the well for 15 min. Then, the wells were washed once with PBS and blocked with blocking buffer (StartingBlock T-20 containing 10 μ g mL⁻¹ herring sperm DNA) for 40 min, followed by drying for 20 min under ambient conditions.

For synthesized oligo detection, the blocked paper wells were washed once with PBS before incubating with target (Tar, 7 nM to 1 mM, Table 1) for 30 min. A minimum of 3 replicates were performed for each target concentration. Then, the wells were washed once with PBS, and 4 μ L reporter probe (Alexa-Tar, Table 1) was added to a final concentration of 300 nM. After 30 min incubation, the wells were washed three times with PBST (PBS with 0.05% Tween-20 (v/v)). The positive control consisted of a biotinylated oligo (b-pos) and Alexa Fluor® 647-oligo (Alexa-pos) (100 nM) pair with complementary sequences, as shown in

Table 1. Thus, positive control wells were left empty during the target incubation step. The fluorescence result was recorded with a LI-COR Odyssey Fc imaging system from LI-COR Biosciences (Lincoln, Nebraska) using a 30 s exposure at 700 nm. The normalized intensity was calculated with ImageJ software by the intensity ratio of each well against the positive control well.

The procedure for cDNA detection was similar to the synthesized oligo detection, except for the sample capture step. Each tube of neutralized cDNA sample was loaded in one well and incubated for 1 h. A minimum of 3 replicates were performed for each cDNA concentration. The capture probe and reporter probe employed for cDNA detection were referred to as b-cDNA and Alexa-cDNA, respectively, as shown in Table 1.

Whole blood clinical sample EBV detection and recovery test

For the whole blood recovery test, increasing numbers of Raji B cells (5×10^5 , 1×10^6 , and 2×10^6) were added to 1 mL of blood (3 replicates for each cell count), and the lymphocytes were separated with Ficoll-Paque PLUS. The experiment was performed according to the manufacturer's instruction. Briefly, cells were added into 1 mL of blood, followed by dilution with 1 mL of PBS. The diluted blood sample was transferred to 1.5 mL Ficoll-Paque PLUS and centrifuged for 40 min at 400g. The lymphocyte layer (middle layer) was transferred to a new tube and washed twice with 2 mL balanced salt solution by centrifuging 10 min at 100g. The remaining lymphocyte layer was lysed with RLT buffer. The RNA extraction, cDNA synthesis, and cDNA detection procedures were identical to those used for the detection of cDNA extracted from Raji B cells. For comparison, three blood samples without spiked cells (negative control) and three samples containing only cells for direct RNA extraction were performed concurrently.

To analyze infected clinical samples, we employed whole blood samples. We performed RNA extraction and followed the same protocol as described earlier to determine the presence and level of EBER-1 RNA in blood samples. For each experiment a minimum of 3 replicates were employed.

Detection directly from infected crude cell lysate

Raji B cells were cultured at 37 °C, 5% CO₂ in RPMI media with 10% fetal bovine serum. Cell counts ranging from 5×10^7 to 5×10^9 cells were lysed in 0.3 mL RLT buffer. Three replicates of each cell lysate were employed in the study. A 12.5 µL aliquot of the cell lysate (ranging from 2.08×10^6 to 2.5×10^8 cells) was utilized as template for reverse transcription by mixing with 0.5 nM RT primer, 2 µL of 10× buffer, 10 µM DTT, 0.5 µM of each dNTP, 100 U M-MLV reverse transcriptase, 40 U RNaseOUT, and RNase-free water to a total volume of 20 µL. The solution was incubated at room temperature for 10 min, followed by RNA hydrolysis at 70 °C for 10 min with 0.33 M NaOH. After neutralization with HCl, the single stranded cDNA was detected with the modified paper microzone. We found the normalized intensity obtained for various cell counts to be comparable to the intensity of the cDNA generated with purified RNA template.

Shelf-life study

To perform this study, RNA samples extracted from Raji B cells were used. The assay was performed using 3 replicates for each day of the study following the same protocol as described above. 200 ng mL⁻¹ of the extracted RNA was employed as the target. The detection was performed after cDNA preparation and followed the protocol for the paper plate assay. The study was repeated at days 1, 2, and 4; weeks 1 and 2; and months 1, 3, and 6.

Results and discussion

Viruses only replicate inside living cells. Except for the unusual case of prions, the starting material for viral replication is DNA or RNA.⁵² Based on the Baltimore classification, viruses fall into seven categories based upon the starting material: dsDNA, ssDNA, dsRNA, (+)ssRNA, (-)ssRNA, ssRNA-RT, and dsRNA-RT.⁵³ Because RNA is a common product of all viral replication strategies, we chose the small non-coding RNA EBER-1, a relatively short nucleotide region (167 nt) (Fig. S1†), as our model target nucleic acid for paper-based detection. However, a reverse transcription step was performed to generate complementary cDNA as the direct target for detection due to the instability of RNA. Typically, special buffer (DEPC-treated or RNase-free buffer) is employed and careful handling is required in RNA detection methods in order to prevent RNA degradation.⁵⁴ In order to reduce this variation, a RT process for cDNA generation was performed prior to detection.

We detected the cDNA target on cellulose paper printed with a 96-well pattern. Compared to prevalent viral nucleic acid detection methods, paper-based detection platforms can provide the basis for POC detection in resource-limited settings because paper is inexpensive, lightweight, disposable, and easy to functionalize and modify.⁵⁵ Also, filter paper is commonly used to store samples and reagents in a dry form for long periods without refrigeration.^{37,56} Therefore, by utilizing paper as the material for our detection platform in combination with oligonucleotides as probes, we were able to impart flexibility to this sensing tool such that each assay step can be stopped as desired and continued at a later time without sacrificing the sensitivity. Furthermore, compared to traditional methods, paper-based detection platforms feature low sampling amount, low detection limit (partially because of the sample concentration on the paper), and short incubation time.⁵⁷

Given the small size of EBER-1, a 10 min reverse transcription (RT) reaction performed at room temperature was sufficient to generate a complete cDNA strand rather than the typical 1 h, 37 °C RT process. Then, the single-stranded cDNA was isolated by hydrolyzing the RNA template with NaOH for 10 min using a boiling water bath. In order to detect the neutralized cDNA, two probes were utilized, a capture probe and a reporting probe – both of which contained complementary sequences to adjacent regions in the target strand. To alleviate the potential for sequence overlaps in the low-temperature hybridization conditions, the capture and reporter probe sequences were carefully chosen to reduce background. Both probe sequences were subjected to multiple Blast alignments, showing limited overlap with human DNA and mRNA sequences (the most likely source of background) as well as with microbial sequences. Additionally, the entire EBER-1 sequence has very limited homology with other microbes and almost no homology with the human genome for the region covered

by the hybridization probes. Two other herpesviruses have similar domains to EBER-1 that show significant homology, but both only infect baboons. Otherwise, no greater than 25% sequence identity is shared with any other annotated sequence. The cDNA target was initially captured by the biotinylated capture probe, which was tethered to the paper surface *via* non-covalent binding of its 3' biotin to physically adsorbed NeutrAvidin® (NA) (Fig. 1). The captured target was detected by hybridization to a reporter probe labeled with Alexa Fluor® 647. The fluorescence intensity was imaged using a LI-COR instrument and analyzed with ImageJ software (Fig. 1).

Characterization of paper platform using synthetic target

Before moving to real-sample detection, we characterized the paper microzone-based detection system first by testing synthetic oligonucleotide target (Tar). 4 μ L aliquots of Tar with concentrations ranging from 7 nM to 1 μ M were detected using this platform. As shown in Fig. 2, a linear calibration curve was obtained in a range of 50–500 nM (0.2 to 2 pmol) from three independent assays each performed with 3 replicates per target concentration. A detection limit of 66.4 nM (265 fmol) was calculated using the standard equation (blank value + 3σ). For normalization, 100% signal was defined as the intensity of Alexa-pos captured by b-pos, while 0% was the background intensity of the negative control (no target addition) (Fig. S2†). All data was analyzed accordingly.

Paper-based detection of EBER-1 from Raji B cells with and without extraction

Next, we evaluated this method by detecting EBER-1 from Raji B cells. Total RNA was extracted from RAji B cells with an RNA isolation kit, the RNA concentration was measured, and three calculated RNA volumes corresponding to different cell counts were added as template for RT. After cDNA synthesis corresponding to the EBER-1 template and RNA hydrolysis, each neutralized mixture containing single-stranded cDNA was added to one well on the paper microzone plate and incubated for 1 h. A calibration plot was prepared that compares fluorescence intensity against the EBER-1 RNA from total extracted RNA. As shown in Fig. 3, there was a significant increase in fluorescence corresponding to higher amounts of total RNA used in the sample. The calculated detection limit was 90.1 ng mL⁻¹, which corresponded to approximately 1.7 nM based on the calculated EBER-1 transcript molecular weight.

We also performed RT using crude cell lysate without RNA extraction and were able to detect the cDNA product with similar sensitivity to the RNA extract samples (Fig. 4), as evident from a detection limit of 4.7×10^5 cells (90 ng mL⁻¹ or ~1.7 nM) that compares favorably to the detection limit obtained using extracted RNA from cells (5.0×10^5 cells). This experiment indicated that the RNA extraction step could be eliminated without adversely affecting the detection of target, thereby removing a significant portion of the essential equipment.

Paper-based detection of EBER-1 from whole blood

To check the utility of our method in the detection of EBV infection, we employed whole blood clinical samples. RNA was extracted from the blood samples for detection and quantification of EBER-1 RNA. A total of 4 samples were evaluated and were positive for

EBV with a concentration range of 61–100 ng mL⁻¹ (~1.1–1.8 nM) of EBER-1 RNA. Additionally, we performed a spike recovery from whole blood. The lymphocyte count for healthy people (older than age 10) is about $1.6\text{--}2.8 \times 10^6$ cells per mL of blood.⁵⁸ As mentioned previously, EBV primarily targets B lymphocytes, and our limit of quantification (LOQ) and limit of detection (LOD) for EBV were about 1.3×10^6 and 5.0×10^5 cells. Due to the likely presence of the EBV genome in humans (>90%), 1 mL of blood was deemed sufficient to account for any matrix effect from latent EBV infection without compromising assay sensitivity. Therefore, we added 1×10^6 (lower than LOQ) and 2×10^6 (higher than LOQ) cells to 1 mL blood and separated the lymphocyte fraction prior to RNA extraction. After RT, the cDNA was detected and the RNA amount was calculated using the RNA calibration curve. Spike recovery was calculated as following:

$$\text{Spike recovery} = \frac{N_{B+C} - N_B}{N_C}$$

N_{B+C} is the calculated total RNA amount of the blood sample with spiked cells. N_B is the RNA amount of negative control (only blood sample), while N_C is the calculated RNA amount by extraction directly from Raji B cells. The recoveries were calculated to be $76.8 \pm 26.2\%$ and $92.2 \pm 10.8\%$ for spikes of 1×10^6 , and 2×10^6 cells in blood, respectively. The low recovery and high inter-assay variation at levels below the quantitation limit may have resulted from RNA degradation during the extraction process (both lymphocyte extraction from blood and RNA extraction from cells). To verify, we performed the RNA extraction procedure using 6 samples over a three-day time period and obtained variations of 17.8% within 6 samples and 22.3% between days for the extraction process. We also spiked synthetic DNA target into the cDNA RT product, which resulted in recoveries of $93.5 \pm 13.5\%$ (lower than LOQ) and $108.6 \pm 15.7\%$ (higher than LOQ) for 0.16 pmol of synthesized DNA target. The reproducibility of this assay was verified with the spike recovery study except for the high standard deviation, which may be inherent to paper-based assays. Whitesides' group tested the paper microzone plate by loading different amounts of FITC-BSA on a plate, followed by fluorescence detection with a spectrophotometer.^{39,40} Their average standard deviation for reproducible loading of reagent on the paper was 10%, which could be partially attributed to inherent variation in paper platform. Therefore, the standard deviation would be much higher for assays that require multiple steps. Due to this limitation of paper-based assays, they are typically considered semi-quantitative. Nevertheless, paper provides an optimal platform for pathogen detection because initial diagnosis requires only a “yes” or “no” response to the presence of pathogen. As such, paper provides a significant asset to effective intervention due to its ability to rapidly and inexpensively screen patients for immediate transition to therapy.

Shelf-life, specificity, and other characteristics of the paper platform

The entire assay protocol can be divided into four intervals: paper modification, sample loading, reporter probe hybridization, and result measurement/image analysis. As stated previously, the protocol can be stopped and our detection platform can be stored after each step. We tested our platform by stopping after the first three intervals and were able to obtain

comparable results as if the assay was completed uninterrupted. After modification with the capture probe, the shelf-life of the paper microzone plate was evaluated for periods of up to 3 months. We performed eight experiments on our paper platform during 3 months and calculated the normalized intensity of each experiment. Compared to the initial measurement on day 1, the variation of normalized intensity was within 20% if the paper microzone plate was stored in dry conditions (Fig. 5). This variation is likely due to the variation in RNA extraction efficiency, which was found to be 17-22% as reported earlier in this manuscript. The results, shown in Fig. 5, when compared to different days, were found to be statistically equivalent, at a 95% confidence interval, using one-way ANOVA followed by Dunnett's multiple comparisons test using GraphPad Prism Version 7.02 for Windows (GraphPad Software, La Jolla California USA, <http://www.graphpad.com>).

We also dried samples on the paper microzone plate prior to detection and performed the detection experiment on the following day without any observable loss of signal intensity as compared to immediate measurement (Fig. 6). This experiment shows that the detection of cDNA can be done at a later time if needed. There was almost no decrease in the fluorescence intensity if the assay was completed and stored without imaging for 3 months (data not shown). Additionally, the selectivity of the system to bind only the target was demonstrated since the random target did not yield a significant signal.

Thus, the assay showed high flexibility, could be performed after interruptions, and could be stored for a period of time prior to signal analysis. All of these factors lend credence to our assertion that this paper-based assay system could provide a viable strategy for POC detection, especially in resource-poor conditions. As this method was only a proof-of-concept platform for paper-based virus detection, further changes can be made to better suit this method for on-site detection. In order to eliminate the need for analytical instrumentation completely, the fluorescent reporter can be replaced with a colorimetric reporter (such as gold or silver nanoparticles) or a UV-excited fluoro-phore. Besides virus detection, this method could be utilized as a general DNA or RNA diagnostic platform. It could also serve as an alternative to traditional Northern blot because of the reduction of experimental steps, smaller sample size, shorter incubation time, and inexpensive reagents.

Conclusion

We developed a paper-based virus detection method that is easily adaptable for clinical laboratory use or POC detection, especially in resource-poor settings, by measuring the RT product from RNA with or without an extraction step. This method enables detection steps that utilize simple equipment, such as water baths and disposable filter paper, while being highly flexible and stable for detection strategies that include immediate or discontinuous analysis. For example, our platform can be stored at room temperature for at least 3 months prior to use, and the result of a completed assay from day 1 could be imaged after 3 months. Additionally, the total cost for each sample was approximately \$1.46 using laboratory pricing; the cost would reduce significantly with scale. Our test also demonstrated easy transfer to clinical sample analysis. Furthermore, overall assay specifications allow it to meet the requirements for employment as a general assay for DNA or RNA detection.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Fig. 1.

Schematic representation of viral RNA detection from cells using paper-based viral RNA detection system. The sample detection procedure required: (a) cell lysis with or without RNA extraction; (b) reverse transcription for 10 min at room temperature; (c) RNA template hydrolysis by addition of 0.33 M NaOH and incubation in a boiling water bath for 10 min; (d) sample loading on the NA-modified paper microzone surface; (e) pre-absorption of NeutrAvidin® (NA) onto the paper surface for immobilization of the biotinylated capture probe (schematically shown as yellow cubes above); (f) sample loading on the NA-coated plate resulting in hybridization between target-derived cDNA target and the capture probe; (g) detection of target hybridization following addition of Alexa Fluor® 647-labeled probe; and (h) result analysis using a LI-COR Odyssey instrument. Increasing concentrations/ volumes of target EBV nucleic acid were indicated as higher fluorescence intensity.

Fig. 2.

Detection of synthesized oligonucleotide target on the paper microzone plate.

Oligonucleotide target was detected within a linear range from 50–500 nM (0.2–2 pmol).

The detection limit was calculated to be 66.4 nM (265 fmol). The data are based on an average response of three independent assays performed using three replicates per target concentration $\pm$ the standard deviation.

Fig. 3. Detection of EBER-1 from the total RNA extracted from Raji B cells employing the paper microzone plate. The data are based on an average response of three samples $\pm$ the standard deviation.

Fig. 4. Result of the detection of RT product from direct cell lysate without RNA extraction. The data are based on an average response of three samples $\pm$ the standard deviation.

Fig. 5. Shelf-life test for the paper microzone plate with immobilized capture probe. The biotin-oligo (b-pos and b-Tar) immobilized paper microzone plate was stored in a desiccator prior to detection. Eight experiments were performed over the course of three months, and the results were analyzed immediately upon assay completion. The resulting normalized intensity was compared with the normalized intensity for day 1 (initial biotin-oligo immobilization). Variation was within 20% for the eight experiments, and no signal decrease was observed. The data are based on an average response of three samples $\pm$ the standard deviation.

Fig. 6.

Detection of pre-loaded sample on the paper microzone. 128 μL aliquots of target (1 nM and 10 nM) and non-target (Ran-Tar, 100 nM and 1 μM) were dried on the paper microzone, followed by hybridization with Alexa-Tar. The target oligo could be easily distinguished even with a much higher concentration of non-target. The data are based on an average response of three samples $\pm$ the standard deviation.

Table 1

Sequences of all probes and synthesized oligonucleotide targets. The first two oligos were used as the complementary positive control pair. The next three oligonucleotides comprised the set of probes for synthesized target detection. The underlined region in the Tar sequence indicates complementarity to b-Tar, while the region in bold corresponds to the Alexa-Tar hybridization site. The bottom two oligos were used as capture probe and reporter probe for cDNA detection

Name	Sequence (5' → 3')
b-pos	CTTAGCTTCCGAGATCAGACGAGA-biotin
Alexa-pos	Alexa Fluor® 647-TCTCGTCTGATCTCGGAAGCTAAG
b-Tar	GCTACAGCCACACACGTCTCCTC-biotin
Tar	<u>GACGTGTGTGGCTGTAGCCACCCGTC</u> CCGGGTACAAGTCCCGGGTGGTGAG
Alexa-Tar	Alexa Fluor® 647-CTCACCACCCGGGACTTGTACCC
b-cDNA	GGGTACAAGTCCCGGGTGGTGAG-biotin
Alexa-cDNA	Alexa Fluor® 647-GAGGAGACGTGTGTGGCTGTAGC